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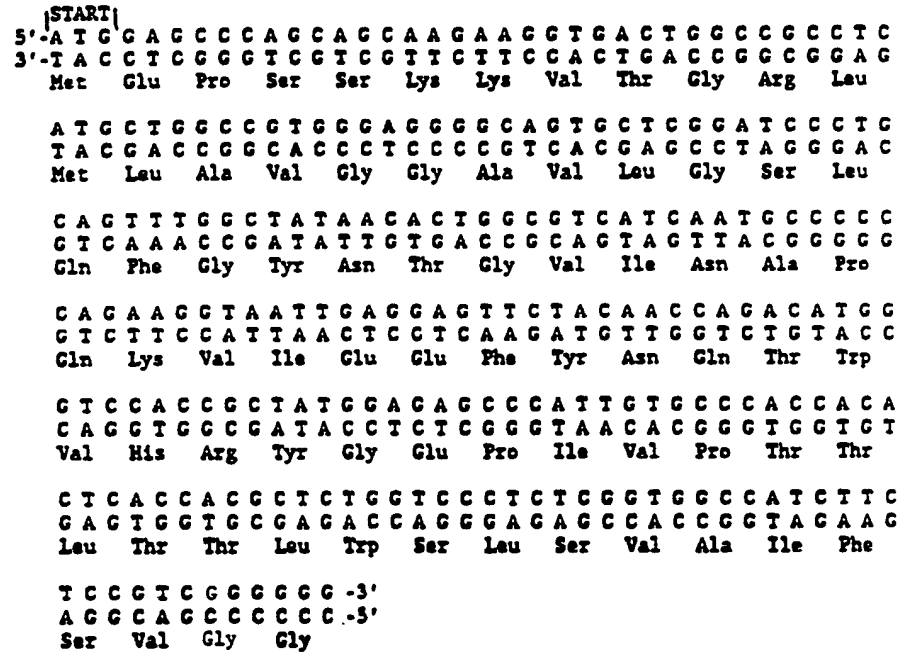

(57) Abstract

A method is provided that comprises transfecting eukary tic cells with a replicable vector comprising a nucleic acid sequence encoding a GTP operably linked to control sequences recognized by the transfected cells. Also provided is an is lated nucleic acid sequence encoding a GTP which sequence includes in part that sh wn in figure 1 or includes that shown in figure 11, as well as vectors and host cell cultures containing such nucleic acid sequence.

* See back of page

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CELLS TRANSFECTED WITH NUCLEIC ACID ENCODING GTP

Background of the Invention

This invention relates to a method for transfecting cells with a vector including nucleic acid encoding a glucose transporter protein (GTP) that enables culturing of the cells in the absence of serum and/or insulin. This invention also relates to the nucleic acid encoding a glucose transporter protein and vectors and host cells containing same.

Description of Related Art

The translocation of glucose across the plasma membrane of virtually all mammalian cells is mediated by a protein transporter ("GTP"). At least two types of GTPs exist: Those that function by a facilitated diffusion (passive) mechanism, and those that utilize an active transport mechanism (Graff et al., J. Cell. Phys., 96:171-188 [1978]; Klip et al., Biochim. Biophys. Acta, 687:265-280 [1982]; Biber and Lienhard, J. Biol. Chem., 261: 16180-16184 [1986]; Weber et al., Fed. Proc., 43:107-112 [1984]; Maiden et al., Nature, 325:641-643 [1987]). The glucose transporter of human erythrocytes, rat adipocytes, and murine adipose cells is hypothesized to transport glucose across the plasma membrane passively (Baldwin and Lienhard, Trends Biochem. Sci., 6:205-208 [1981]; Czech, Mol. Cell. Biochem., 11:51-63 [1976]), whereas the glucose transporter of epithelial cells functions by an active transport mechanism (Moran et al., J. Biol. Chem., 258:15087-15090 [1983]). Other cells such as L6 rat myoblasts and chick embryo fibroblasts may contain both types of glucose transporters (D'Amore and Lo, J. Cell. Phys., 127:95-105 [1986]; Christopher et al., Biochem. J., 158:439-450 [1976]).

Studies of the passive GTP from various cell types have provided information regarding its molecular and biochemical characteristics. Western blot analysis of the protein from 3T3-21 adipocytes reveals a molecular weight of approximately 55,000 daltons (Biber and Lienhard, supra), which is consistent with the molecular weight of the human erythrocyte and the murine glucose transporters (also approximately 55,000 daltons). These proteins are related immunologically, and appear to be glycosylated. The primary translocation product of both murine and human mRNAs is about 38,000 daltons (Haspel et al., J. Biol. Chem., 260:7219-7225 [1985]). The nucleotide sequences of the rat brain glucose transporter cDNA (Mueckler et al., Science, 229:941-945 [1985]) and a glucose transporter cDNA from a human hepatoma library (Birnbaum et al., Proc. Natl. Acad. Sci. USA, 83:5784-5788 [1986]) are very similar and suggest that 97 percent of the amino acid residues are identical. The cDNA of rabbit brain GTP has also been reported (Asano et al., Biochem. Biophys. Res. Commun., 154:1204-1211 [1988]). A hydropathic profile of the passive glucose transporter of human erythrocytes suggests that the protein has twelve membrane-spanning regions and that both the amino and carboxyl termini are on the cytoplasmic face of the cell.

Many mitogenic events are known to increase the transport of glucose into cells. Serum is required for growth of many cell lines in culture and is also well known for its stimulation of glucose uptake. Kalckar et al., Proc. Natl. Acad. Sci. USA, 77:5958-5961 (1980). Insulin appears to be the predominant hormone of serum responsible for this effect (Morgan and Whitfield, In Current Topics of Membranes and Transport, Bronner and Klenzeller, eds., Academic Press, New York, N.Y., Vol. 4, pp. 255-303 [1974]; Clausen, T., in Current Topics of Membranes and Transport, Bronner and Kleinzeller, eds., Academic Press, New York, N.Y., Vol. 6, pp. 169-226 [1975]).

One current theory to explain insulin stimulation of glucose transport in fat and muscle cells is that insulin enhances translocation of a "reserve pool" of glucose transporters located intracellularly to the plasma membrane (Cushman and Wardzala, J. Biol. Chem., 255:4758-4762 [1980]; Kono et al., J. Biol. Chem., 256:6400-6407 [1981]; James et al., J. Biol. Chem., 262:11817-11824 [1987]; Kono et al., J. Biol. Chem., 257:10942-10947 [1982]; Oka and Czech, J. Biol. Chem., 259:8125-8133 [1984]; Ezaki et al., J. Biol. Chem., 261:3295-3305 [1986]). Subcellular fractionation of the cells indicates that the GTP is located in both the heavy (plasma membrane) and the light microsome fractions. If cells are subjected to insulin stimulation prior to membrane fractionation, a progressive increase in the amount of GTP is seen in the plasma membrane fraction with a corresponding decrease of GTP in the light microsome fraction. Dexamethasone has been shown to reverse this translocation process; however, the effect of dexamethasone can be overcome by addition of insulin (Horner et al., J. Biol. Chem., 262: 17696-17702 [1987]).

Exogenous application of either transforming growth factor-β or epidermal growth factor to cultured 3T3 cells, NRIL-49 rat kidney cells, or human fibroblasts has been shown to increase the transport of glucose (Barnes and Colowick, J. Cell. Phys., 89:633-640 [1976]; Inman and Colowick, Proc. Natl. Acad. Sci. USA, 82:1346-1349 [1985]). An increase in glucose transporter mRNA synthesis has been demonstrated to occur in fibroblasts exposed to either fibroblast growth factor, platelet-derived growth factor, epidermal growth factor, serum, or phorbol esters (Hiraki et al., J. Biol. Chem., 263:13655-13662 [1988]). Platelet-derived growth factor has been found to induce both glucose transport and the amount of membrane-associated GTP in mouse fibroblasts (Rollins et al., J. Biol. Chem., 263:16523-16526 [1988]). Phorbol esters also stimulate the transport of glucose in rat adipocytes (Martz et al., J. Biol. Chem., 261:13606-13609 [1986]) and chicken embryo fibroblasts (Yamada et al., J. Cell. Phys., 127:211-215 [1986]). The increase in transport correlates well with an increased number of functioning transporters in the plasma membrane. Viral transformation of cells in culture has also been shown to increase the transport of glucose. Normal mouse embryo cells and chicken embryo fibroblasts both demonstrate this response after transformation with murine sarcoma virus and Rous sarcoma virus, respectively (Hatanaka et al., J. Natl. Cancer Inst., 43:1091-1096 [1969]; Hatanaka, Biochim. Biophys. Acta, 355:77-

104 [1974]; Lang and Weber, J. Cell. Phys., 94:315-320 [1978]). Transfection of cells with the src oncogene has been shown to increase glucose transport by four- to five-fold (Shawver et al., Mol. and Cell. Biol., 7:2112-2118 [1987]; White and Weber, Mol. and Cell. Biol., 8:138-144 [1988]). The src oncogene increases the number of functioning glucose transporters in the membranes of both chicken embryo fibroblasts and rat-1 cells. Transformation of normal, anchorage-dependent rat liver epithelial cells with an inducible ras gene has been demonstrated to cause several phenotypic alterations, including loss of contact inhibition of growth, an increase in glucose transport, anchorage-independent growth, and diminished lag phase of the growth curve (Huber and Cordingley, Oncogene, 3:245-256 [1988]). Differences in growth kinetics and glucose transport could be directly correlated with the levels of ras expression.

Only a small amount of human hepatoma-type glucose transporter mRNA has been observed in insulin-sensitive tissues such as adipocytes and muscle (Flier et al., J. Clin. Invest., 79:657-661 [1987]). These results suggest the presence of other types of GTPs in these tissues and also raise the issue of whether insulin mediation of glucose transport activity depends on a tissue-specific glucose transporter or a tissue-specific signaling mechanism. Site-directed antibodies against the human hepatoma-type glucose transporter have revealed that a small amount of the human hepatoma-type GTP exists in rat adipocytes, which decreases by approximately 50% in the low density microsomes in response to insulin in a manner similar to insulin-induced translocation of a large amount of other types of rat adipocyte glucose transporters (Oka et al., J. Biol. Chem., 263:13432-13439 [1988]).

Recombinant desired polypeptides of medical therapeutic or diagnostic value commonly are produced by host cells that require extremely expensive, complex media. There is a need in the art for eukaryotic cells that do not require insulin (which is contained in serum) or other expensive defined growth factors for growth and survival of the cells.

Accordingly, it is an object of the present invention to modify eukaryotic cells that normally require serum for survival and growth such that they grow without serum or any protein growth factor ordinarily required for cell survival or growth.

This and other objects will become apparent to one of ordinary skill in the art.

Summary of the Invention

These objects are achieved by the provision of a method comprising transfecting eukaryotic cells with a replicable vector comprising a nucleic acid sequence encoding a GTP operably linked to control sequences recognized by the transfected cells. Moreover, the invention provides a eukaryotic cell comprising a replicable vector comprising a nucleic acid sequence encoding a GTP operably linked to control sequences recognized by the eukaryotic cells.

In another aspect, this invention relates to an isolated nucleic acid sequence encoding a GTP, which sequence comprises at its 5' end the nucleotide sequence shown in Fig. 1,

where the start ATG codon is indicated, or comprises the nucleotide sequence shown in Fig. 11, where the start and stop codons are indicated, and optionally further comprises the sequence upstream of the start codon shown in Figure 9A as the "cho" sequence. Also provided are an expression vector comprising this nucleic acid sequence operably linked to control sequences recognized by a host transformed by the vector and host cells transformed with the vector.

In yet another aspect, the invention provides an isolated eukaryotic cell having its origin in a multicellular organism, which cell constitutively expresses GTP so that the cell is not dependent upon serum, insulin, or transferrin for growth or survival.

The benefit of the invention herein is achieved when eukaryotic cells transfected with nucleic acid encoding a GTP are grown in culture medium that is free of protein growth factors, fatty acids, metal ions, transferrin, serum and/or insulin otherwise necessary for survival and growth of the cells. These cells are characterized by expression of GTP at levels that are sufficiently constitutive as to free the cells from dependence on one or more otherwise required growth factors. In addition, the DNA sequence encoding a novel GTP from Chinese hamster ovary cells useful in this method has been isolated and sequenced.

Brief Description of the Drawings

Figure 1 depicts the partial nucleotide sequence of cDNA encoding a GTP from Chinese hamster ovary cells at the 5' end and depicts a portion of the predicted mature protein sequence at the N-terminus.

Figure 2 depicts the construction of expression plasmid pRK.GTP (coding for a GTP).

Figure 3 depicts the construction of expression plasmid pFD11 (coding for DHFR).

Figure 4 depicts the construction of expression plasmid pRK.t-PA.

Figure 5 depicts the growth (A) and viability (B) of cells cotransfected with pFD11 and pRK.GTP and grown in medium supplemented with transferrin, trace elements, linoleic acid-ethanol, and insulin.

Figure 6 depicts the growth (A) and viability (B) of cells cotransfected with pFD11 and pRK.GTP and grown in medium supplemented with transferrin, trace elements, and linoleic acid-ethanol.

Figure 7 depicts the growth (A) and viability (B) of cells cotransfected with pFD11 and pRK.GTP and grown in medium supplemented with transferrin and trace elements.

Figure 8 depicts the growth (A) and viability (B) of cells cotransfected with pFD11 and pRK.GTP and grown in medium containing no supplements.

Figure 9 (A-H) depicts the nucleotide sequences of the rat (Mueckler et al., supra), human (Birnbaum et al., supra), and CHO DNA; the homologies are indicated by boxed-in regions.

Figure 10 (A-B) depicts the amino acid sequence of the Chinese hamster ovary (cho), rat, and human (hum) GTP using the one-letter amino acid code; the homologies are

indicated by boxed-in regions.

Figure 11 (A-E) depicts the full-length nucleotide sequence of the cDNA encoding a GTP from Chinese hamster ovary cells. The amino acids encoded by the DNA are indicated directly below the sequence using the three-letter code, and both start and stop codons are indicated. Both 5' and 3' untranslated sequences are also shown.

Description of the Preferred Embodiments

A. Definitions

As used herein, the term "GTP" refers to a protein, whether having the sequence of the native molecule or being a derivative or amino acid sequence variant thereof, that mediates the transport of glucose across the plasma membrane of cells, whether by a facilitated diffusion or an active transport process. The protein, which generally has an average molecular weight of about 55,000, is suitably from any eukaryotic species and cell source, such as, e.g., from human hepatoma or erythrocytes, rat adipocytes, rat brain, rabbit brain, epithelium, rat myoblasts, Chinese hamster ovary cells, and chick embryo fibroblasts.

Derivatives and amino acid sequence variants are defined as molecules in which the amino acid sequence, or other feature of a native GTP, has been modified covalently or noncovalently. Amino acid sequence variants include not only alleles of the native sequence, but also predetermined mutations thereof. Generally, amino acid sequence variants have an amino acid sequence with at least about 80% homology, and more typically at least about 90% homology, to that of a native GTP, for example, the one encoded by the nucleotide sequence shown in Fig. 11. Henceforth, the term "GTP" shall mean any one of the native sequences or a variant form unless otherwise appropriate.

Thus, included within the scope of the transfection method of the present invention is a GTP having a native sequence, especially that from Chinese hamster ovary cells encoded by the complete sequence in Figure 11, analogous GTPs from other microbial, vertebrate, or invertebrate eukaryotic species such as insect, human, bovine, equine, rabbit, rat, porcine, ovine, canine, murine, and feline species, and biologically active amino acid sequence variants of these proteins, including alleles and in vitro-generated covalent derivatives of GTPs that demonstrate the protein's activity.

As used herein, the term "eukaryotic cells" refers to eukaryotic cells capable of growth in culture and of expressing the gene encoding a desired polypeptide. Such cells may be vertebrate or invertebrate, such as a yeast, fungal, or mammalian host. Preferably, the cells are those that require regulation of transmembrane glucose transport, more specifically those that in the presence of basal (i.e., normal) levels of the GTP (i.e., those that are not transfected) require serum, insulin, and linoleic acid or other lipid source for serial culture or growth. Suitable hosts include, e.g., Chinese hamster ovary cells, bovine kidney cells, insect cells, N51 cells, VERO cells, HeLa cells, human embryonic kidney cells (293), W138,

COS-7, MDCK cells, BHK (baby hamster kidney), MRC5, FS4, Hep G2 (human hepatoma line), mouse L cells, mouse 3T3 cells, primary spleen cells, Namalwa cells, and myelomas.

Alternatively, the eukaryotic cells are hybridoma cells. Hybridoma cells are generally obtained according to the somatic cell hybridization procedure described by Milstein et al., Nature, 256:495-497 [1975] and Kohler et al., Eur. J. Immunol., 6:511-519 [1976]. Basically, in this procedure, a mouse or other appropriate host animal is injected with an immunogen and then sacrificed. The resulting antibody-producing cells, taken, e.g., from its spleen or lymphoid tissue, are fused with appropriate selectable cancer (myeloma) cells using a suitable fusogen such as polyethylene glycol to form a hybridoma. The transfection with the GTP is preferably conducted after the fusion takes place, but optionally one can transfect the myeloma cells or antibody-producing cells before fusion thereof.

Preferred myeloma cells are those that fuse efficiently, support stable high-level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center in San Diego, Calif. After the fusion, the hybridoma cells thus prepared are washed to remove the fusogen and then seeded and grown in a selective growth medium such as HAT to select only those hybridomas resistant to the medium and which are considered to be immortal.

The hybridomas thus selected are screened for production of individual antibodies directed against the specific antigens desired, using techniques such as, e.g., radioimmunoassay and/or enzyme immunoassay, and also may be screened for affinity to the antigen of interest. After such screening to isolate individual clones that secrete the antibodies desired, the selected clones are preferably transfected with the vector encoding the GTP. The positive clones may be subcloned by limiting dilution procedures, and grown by known procedures. The monoclonal antibodies secreted by the subclones may be separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, e.g., ammonium sulfate precipitation, gel electrophoresis, dialysis, DEAE cellulose chromatography, or affinity chormatography.

As used herein, the term "desired polypeptide" refers to any polypeptide intended to be produced in a host cell, but which the host cell either normally does not produce itself or produces in small amounts, and which is not normally necessary for the continued existence of the cells. The desired polypeptide includes a polypeptide having as few as about five amino acids to much larger proteins such as factor VIII. Such a polypeptide includes any molecule having the pre- or prepro-amino acid sequence, as well as amino acid or glycosylation variants (including natural alleles) capable of exhibiting a biological activity in common with the desired protein. The term also includes, but is not limited to, polypeptides that are advantageously produced in eukaryotic systems, e.g., those that are

glycosylated in the native state or those that cannot be readily expressed, or readily secreted without degradation, in prokaryotic cells. Preferably the polypeptide is heterologous to the host cells in which it is made and is a human polypeptide.

Examples of such desired polypeptides include mammalian polypeptides, such as, e.g., a growth hormone, including human growth hormone, des-N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroid stimulating hormone; thyroxine; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; leutinizing hormone; glucagon; factor VIII; an antibody; lung surfactant; a plasminogen activator, such as urokinase or human tissue-type plasminogen activator (t-PA); bombesin; factor IX, thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; a serum albumin such as human serum albumin; mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; tissue factor protein; inhibin; activin; vascular endothelial growth factor; receptors for hormones or growth factors; integrin; thrombopoietin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), or a nerve growth factor such as NGF-β; platelet-derived growth factor; fibroblast growth factor such as aFGF and bFGF; epidermal growth factor; transforming growth factor (TGF) such as TGF-alpha and TGF-beta; insulin-like growth factor-I and -II; insulin-like growth factor binding proteins; CD-4; erythropoietin; osteoinductive factors; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1, IL-2, IL-3, IL-4, etc.; superoxide dismutase; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; and fragments of any of the above-listed polypeptides. In addition, one or more predetermined amino acid residues on the polypeptide may be substituted, inserted, or deleted, if the expression levels of the resulting variants are not significantly reduced.

Preferably, the desired polypeptide is transforming growth factor-beta, CD-4, transforming growth factor-alpha, activin, tissue plasminogen activator, DNase, insulin-like growth factor, or nerve growth factor.

As used herein, the expression "protein growth factors, fatty acids, or metal salts ordinarily required for cell growth" refers to proteins, fatty acids, and metal salts that are ordinarily added to the culture media for growth of cells. Examples of such substances include insulin, transferrin, fatty acids such as linoleic acid and linolenic acid, and trace elements such as selenous acid, manganese salts, nickel salts, stannous salts, zinc salts, copper salts, ferrous salts, hydrochloric acid, metasilicates, paramolybdates, and vanadium oxides. Obviously, the amounts of each of these substances required for growth or survival, as well as the particular one required, will vary from cell to cell, as is readily apparent to one of

ordinary skill in the art.

As used herein, the term "selectable marker" refers to a marker that allow the transfected cells to be identified readily. Generally such selectable markers include those in which amplification can be detected by exposing host cells to changes in the "selection agent." Such selectable markers usually require a parental cell line that is genotypically deficient in the selection gene. Examples include the genes for hydroxymethylglutanyl CoA reductase (Sinensky, Biochm. Biophys. Res. Commun., 78:863 [1977]), ribonucleotide reductase (Meuth et al., Cell, 3:367 [1943]), aspartate transcarbamylase (Kemp et al., Cell, 9:541 [1976]), adenylate deaminase (DeBatisse et al., Mol. and Cell Biol., 2:1346-1353 [1982]), mouse dihydrofolate reductase (DHFR), and, with a defective promoter, mouse thymidine kinase (TK). Additionally, asparagine synthetase has been used successfully as a selectable marker (Cartier et al., Mol. Cell. Biol., 7:1623-1628 [1987]).

Selectable markers also include those that are expressed in transformants regardless of the genotype of the parental cell. Examples of such markers include the genes for prokaryotic enzymes such as xanthine-guanine phosphoribosyltransferase (Mulligan et al., Proc. Natl. Acad. Sci. USA, 78: 2072-2076 [1981]) and aminoglycoside 3'-phosphotransferase (Colbere-Garapin et al., J. Mol. Biol., 150:1-14 [1981]).

Examples of dominant selection markers that are also detectably amplified include the mutant DHFR gene described by Haber et al., Somatic Cell Genet., 4:499-508 [1982], cell surface markers such as HLA antigens and genes coding for enzymes such as specific esterases that produce fluorescent or colored products from fluorogenic or chromogenic substrates. Preferred herein as a selectable marker is DHFR, and the preferred selection agent for its detection is methotrexate or any other inhibitor of DHFR activity such as other folic acid analogs, or a selection medium lacking glycine, hypoxanthine, and thymidine (GHT).

"Transfection" refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

"Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S.N. Proc. Natl. Acad. Sci. (USA), 69:2110 [1972] and Mandel *et al.*, J. Mol. Biol. 53:154 [1970], is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with Agrobacterium tumefaciens is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 [1983] and WO 89/05859 published June 29, 1989.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham, F. and van der Eb, A., Virology, 52:456-457 [1978] is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Pat. No. 4,399,216 issued August 16, 1983. Transformations into yeast are typically carried out according to the method of Van Solingen, P., et al., J. Bact., 130:946 [1977] and Hsiao, C.L., et al., Proc. Natl. Acad. Sci. (USA) 76:3829 [1979]. However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used.

It will be understood that other methods for enhancing the expression of GTP, other than transfecting the protein, can be used to accomplish the objectives of this invention. For example, cell treatments that enhance the expression of endogenous GTP, e.g. by activation of the endogenous promoter/operator for the protein, are equivalents to the transfection embodiment described herein.

As used herein, the term "isolated DNA" is understood to mean chemically synthesized DNA, cDNA, chromosomal, or extrachromosomal DNA with or without the 3'- and/or 5'-flanking regions, but preferably with such flanking ends to ensure that the regulatory portions of the protein are present.

B. Modes for Carrying out the Invention

1. Transfection Method

The cells to be transfected with a vector encoding a GTP are suitably any eukaryotic cell as defined above. This includes eukaryotic microbes, such as fungi (e.g., A. awamori and A. niger) and yeast cultures. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available, such as Schizosaccharomyces pombe (EP 139,383 published May 2, 1985), Saccharomycopsis lipolytica (EP 320,994 published June 21, 1989), S. uvarum, S. carlsbergensis, or mixtures thereof. For expression in Saccharomyces, the plasmid YRp7, for example (Stinchcomb et al., Nature, 282:39 [1979]; Kingsman et al., Gene, 7:141 [1979]; Tschemper et al., Gene, 10:157 [1980]), is commonly used. This plasmid already contains the trp1 gene that provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44,076 or PEP4-1 (Jones, Genetics, 85:12 [1977]). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem., 255:2073 [1980]) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg., 7:149 [1968]; Holland et al., Biochemistry, 17:4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-

phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, an origin of replication, and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as Spodoptera frugiperda (catapillar), Aedes aegypti (mosquito), Aedes albopictus (mosquito), Drosphila melanogaster (fruitfly), and Bombyx mori host cells have been identified. See, e.g., Luckow et al., Bio/Technology, 6:47-55 [1988); Miller et al., in Genetic Engineering, Setlow, J.K. et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., Nature, 315:592-594 [1985]. A variety of such viral strains are publicly available, e.g., the L-1 variant of Autographa californica NPV and the Bm-5 strain of Bombyx mori NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of Spodoptera frugiperda cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can readily be utilized as hosts for genes encoding many heterologous proteins. Typically, plant cells are transfected by incubation with certain strains of the bacterium Agrobacterium tumefaciens, which has been previously manipulated to contain the heterologous gene (protein) of interest. During incubation of the plant cell culture with A. tumefaciens, the DNA encoding the heterologous protein is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express this heterologous protein. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker et al., J. Mol. Appl. Gen., 1:561 [1982]. In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. EP 321,196 published June 21, 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [Tissue Culture, Academic Press, Kruse and Patterson, editors [1973)]. Examples of such useful host

secondary coding sequence (selectable marker) comprises dihydrofolat reductase (DHFR) that is affected by an externally controlled parameter, such as methotrexate (MTX) or a selection medium lacking glycine, hypoxanthine, and thymidine (GHT), thus permitting control of expression by control of the MTX or GHT concentration. The selectable marker may be on the same vector as the DNA encoding the GTP, may be contained on a different vector that is used to cotransfect the eukaryotic host, or may be integrated into the host chromosome.

In selecting a preferred host cell for transfection by the vectors of the invention that comprise DNA sequences encoding both GTP and DHFR protein, it is appropriate to select the host according to the type of DHFR protein employed. If wild-type DHFR protein is employed, it is preferable to select a host cell that is deficient in DHFR, thus permitting the use of the DHFR coding sequence as a marker for successful transfection in selection medium that lacks hypoxanthine, glycine, and thymidine. An appropriate host cell in this case is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. (USA) 77:4216 [1980].

On the other hand, if DHFR protein with low binding affinity for MTX is used as the controlling sequence, it is not necessary to use DHFR-deficient cells. Because the mutant DHFR is resistant to methotrexate, MTX-containing media can be used as a means of selection provided that the host cells are themselves methotrexate sensitive. Most eukaryotic cells that are capable of absorbing MTX appear to be methotrexate sensitive. One such useful cell line is a CHO line, CHO-K1 (ATCC No. CCL 61).

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to prepare the plasmids required.

If blunt ends are required, the preparation may be treated for 15 minutes at 15°C with 10 units of Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments may be performed using 6 percent polyacrylamide gel described by Goeddel et al., Nucleic Acids Res., 8:4057 [1980].

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are typically used to transform E. coli K12 strain MM294 (ATCC 31,446) or other suitable E. coli strains, and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared and analyzed by restriction mapping and/or DNA sequencing by the method of Messing et al., Nucleic Acids Res., 9:309 [1981) or by the method of Maxam et al., Methods of Enzymology, 65:499 [1980).

After introduction of the DNA into the mammalian cell host and selection in medium for stable transfectants, amplification of mutant DHFR-protein-coding sequences is effected

by growing host cell cultures in the presence of approximately 20 nM to 1 μM concentrations of methotrexate, a competitive inhibitor of DHFR activity. The effective range of concentration is highly dependent, of course, upon the nature of the DHFR gene and the characteristics of the host. Clearly, generally defined upper and lower limits cannot be ascertained. Suitable concentrations of other folic acid analogs or other compounds that inhibit DHFR could also be used. MTX itself is, however, convenient, readily available, and effective.

In another embodiment, DNA encoding a desired polypeptide as defined above is incorporated into the same vector as the DNA encoding the GTP and/or selectable marker. Alternatively, and preferably, the DNA encoding the desired polypeptide is on a separate expression vector used to cotransfect the eukaryotic cells along with the other vector(s) containing the GTP and selectable marker genes. Construction of suitable vectors containing the DNA encoding the desired polypeptide and control sequences employs standard ligation techniques, and isolated plasmids or DNA fragments are cleaved, tailored, and religated to obtain the desired replicable vector. Achievement of blunt ends, size separation of cleaved fragments, and sequence analysis, and use of other techniques for preparing the appropriate vector, is suitably as described above in connection with DHFR vectors and below in connection with generally known techniques.

The GTP useful herein for transfection or transformation of the eukaryotic cells is suitably any GTP as defined above, including amino acid sequence variants, whether prepared covalently or by mutations in the DNA. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the native amino acid sequence. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure (see EP 75,444A).

At the genetic level, these variants ordinarily are prepared by site-directed mutagenesis of nucleotides in the DNA encoding the GTP, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. The variants typically exhibit the same qualitative biological activity as the naturally occurring analog.

While the site for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example, site-specific mutagenesis.

2. Recombinant Expression of DNA encoding Chinese Hamster Ovary Cell Derived GTP

The GTP molecule whose complete amino acid sequence is shown in Figur 11 is synthesized in recombinant cell culture. For such synthesis, it is first necessary to secure nucleic acid that encodes a GTP. DNA encoding a GTP molecule is obtained from Chinese hamster ovary cells by (a) obtaining a DNA library from the appropriate cell line, (b) conducting hybridization analysis with labeled DNA encoding the GTP or fragments thereof (up to or more than 100 base pairs in length) to detect clones in the library containing homologous sequences, and (c) analyzing the clones by restriction enzyme analysis and nucleic acid sequencing to identify full-length clones. DNA that is capable of hybridizing to a glucose-transporter-protein-encoding cDNA under stringent conditions is useful for identifying DNA encoding the particular GTP desired. Suitable such conditions are described further below. Full-length clones were present in the cDNA library screened herein. Alternatively, genomic libraries will provide the desired DNA. The complete sequence of the Chinese hamster ovary cell cDNA that was ultimately determined is shown in Fig. 11. This sequence is derived from the sequencing gel of plasmid pRK.GTP, which is transfected into the cell line GTP-S1 deposited as ATCC number CRL 10,208.

Once this DNA has been identified and isolated from the library it is ligated into a replicable vector for further cloning or for expression.

In one example of a recombinant expression system a GTP is expressed in eukaryotes by transforming with an expression vector comprising DNA encoding the protein. It is preferable to transform host cells capable of accomplishing such processing so as to localize the protein to the plasma membrane of the host cell if possible.

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of eukaryotic organisms.

In general, of course, prokaryotes are preferred for the initial cloning and sequencing of DNA sequences and construction of the vectors useful in the invention. For example, E. coli K12 strain MM 294 (ATCC No. 31,446) and JM101 (Messing et al., Nucl. Acid Res., 9:309 [1981]) or its lambda- minus derivative SR101 are particularly useful. Other microbial strains that may be used include E. coli strains such as E. coli B, E. coli X1776 (ATCC No. 31,537), and E. coli DH-1 (ATCC No. 33,849). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes may also be used for expression. The aforementioned strains, as well as E. coli strains W3110 (F-, lambda-, prototrophic, ATCC No. 27,325), and K5772 (ATCC No. 53,635), bacilli such as Bacillus subtilis, and other enterobacteriaceae such as Salmonella typhimurium or Serratia marcesans, and various Pseudomonas species, such as Pseudomonas aeruginosa, may be used. Examples of hosts that are capable of converting glucose or other commonly available metabolites to 2, 5 - DKG are those from the genera Acetobacter,

Gluconobacter, Acetomonas, and Erwina. Examples of particular hosts include Erwinia herbicola (ATCC No. 21,998), Acetobacter oxydans subspecies melanogenes (ATCC No. 9937), Acetobacter cerinus (IFO 3263 IFO 3266), Gluconobacter rubiginosus (IFO 3244), Acetobacter fragum (ATCC No. 21,909), and Acetobacter suboxydans subspecies industrious (ATCC No. 23,776). Eubacteria are preferred of the prokaryotic hosts.

In general, plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences that are capable of providing phenotypic selection in transformed cells. For example, E. coli is typically transformed using pBR322, a plasmid derived from an E. coli species (see, e.g., Bolivar et al., Gene, 2:95 [1977]). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid or phage, must also contain, or be modified to contain, promoters that can be used by the microbial organism for expression of its own proteins.

Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (Chang et al., Nature, 375:615 [1978]; Itakura et al., Science, 198:1056 [1977]; Goeddel et al., Nature, 281:544 [1979]) and a tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res., 8:4057 [1980]; EPO Appl. Publ. No. 0036,776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (see, e.g., Siebenlist et al., Cell, 20:269 [1980]).

In addition to prokaryotes, eukaryotic hosts may be employed, using the host and vectors systems mentioned above in the context of the transfection system.

In order to simplify the examples and claims, certain frequently occurring methods will be referenced by shorthand phrases.

"Site-directed mutagenesis" is a technique standard in the art, and is conducted using a synthetic oligonucleotide primer complementary to a single-stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells that harbor the phage. The plaques are hybridized with kinased synthetic primer at a temperature that permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques that hybridize with the probe are then selected and cultured, and the DNA is recovered.

"Operably linked" refers to juxtaposition such that the normal function of the

components can be performed. Thus, a coding sequence "operably linked" to control sequences refers to a configuration wherein the coding sequence can be expressed under the control of these sequences and wherein the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

"Control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

"Expression system" refers to DNA sequences containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed with these sequences are capable of producing the encoded proteins. To effect transformation, the expression system may be included on a vector; however, the relevant DNA may then also be integrated into the host chromosome.

As used herein, "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, "transformants" or "transformed cells" includes the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same functionality as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed from such available plasmids in accord with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

"Digestion," "restriction endonuclease digestion," or "endonuclease digestion" of DNA refers to catalytic cleavage of the DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction enzymes, and the sites for which each is specific are called restriction sites. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements as

established by the enzyme suppliers are used. Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters representing the microorganism from which each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1 μg of plasmid or DNA fragment is used with about 1-2 units of enzyme in about 20 μl of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation of about 1 hour at 37°C is ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme infrequently is followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional (T. Maniatis et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory, 1982) pp. 133-134).

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see R. Lawn et al., Nucleic Acids Res. 9:6103-6114 [1981], and D. Goeddel et al., Nucleic Acids Res. 8:4057 [1980].

"Southern Blot Analysis" is a method by which the presence of DNA sequences in a digest or DNA-containing composition is confirmed by hybridization to a known, labelled oligonucleotide or DNA fragment. For the purposes herein, unless otherwise provided, Southern blot analysis shall mean separation of digests on 1 percent agarose, and transfer to nitrocellulose or a nylon membrane by the method of E. Southern, J. Mol. Biol. 98:503-517 [1975] or Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory, 1989), and hybridization as described by T. Maniatis et al., Cell 15:687-701 [1978] or Sambrook et al., supra.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (T. Maniatis et al., 1982, supra, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

"Preparation" of DNA from transformants means isolating plasmid DNA from

microbial culture. Unless otherwise provided, the alkaline/SDS method of Maniatis et al., 1982, supra, p. 90, may be used.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid phase techniques such as described in EP Pat. Pub. No. 266,032 published May 4, 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler et al., Nucl. Acids Res., 14:5399-5407 [1986]). They are then purified on polyacrylamide gels.

The following examples are intended to illustrate the best mode now known for practicing the invention, but the invention is not to be considered limited thereto.

Example 1

Cloning of GTP cDNA

A. Construction of a CHO DNA Library

Total cellular RNA from Chinese hamster ovary-FR4 cells (CHO-FR4) was extracted as follows: Approximately 8 x 10^8 CHO-FR4 cells were grown in a medium prepared as follows: A total of 6.69 g of Dulbecco's Minimum Essential Medium (ATCC Media Handbook, First Ed., 1984, p. 77) and 5.31 g Ham's F12 (ATCC Media Handbook, First Ed., 1984, p. 78) but without glycine, hypoxanthine, and thymidine were mixed together. A total of 2.44 g of sodium bicarbonate and 0.055 g of sodium pyruvate were added to the mixture, followed by purified water to a volume of 1 liter to dissolve the components. The pH was then checked and adjusted to 6.95 with liquid 6N HCl. The medium was then filter sterilized. Just before use was added 70-75 cc of fetal bovine serum (FBS) (which was diafiltered) to obtain a serum concentration in the medium of 7-7.5 percent. Finally, 0.5 μM methotrexate was added.

The cells were lysed in a buffer containing 10 mM NaCl, 10 mM Tris pH 7.5, 1.5 mM $MgCl_2$ and 1% (v/v) Nonidet-40 detergent. The nuclei and cellular debris were pelleted and discarded, the supernatant was extracted with phenol and chloroform, and the RNA was precipitated with ethanol using standard procedures (Maniatis et al., 1982, supra). Approximately 4 milligrams of RNA were incubated with oligo(dT) cellulose in 20 mM Tris pH 7.5, 0.5M LiCl, 1 mM EDTA and 0.1% SDS, and the poly(A)$^+$ RNA which bound to the oligo(dT) cellulose was eluted with a buffer consisting of 10 mM Tris pH 7.6 and 1 mM EDTA. Approximately 50 micrograms of poly(A)$^+$ RNA were obtained, and five micrograms of this poly(A)$^+$ RNA were used for cDNA synthesis.

A kit purchased from the Amersham Corporation (catalog number RPN.1256) was used for cDNA synthesis. Five micrograms of poly (A)$^+$ RNA were primed with oligo(dT) and the first strand of cDNA was generated using reverse transcriptase. Approximately 1.3 micrograms of cDNA were obtained. The RNA strand of the cDNA/RNA heteroduplex was then partially digested using E. coli ribonuclease H, and the second strand of DNA was

synthesized with E. coli DNA polymerase I using the nicked RNAs as primers. The ends of the double-stranded DNAs were blunted using T4 DNA polymerase, and synthetic oligonucleotide linkers (5'AATTCGTCTAGACGTCGAC and 5'GTCGACGTCTAGACG, which correspond to the restriction sites EcoRI-XbaI-SalI) were ligated to approximately 700 nanograms of double-stranded cDNA using the procedure of Maniatis et al., 1982, supra. The cDNA was then fractionated on a 5% polyacrylamide gel. A gel slice containing the cDNA larger than about 500 base-pairs was cut out and the cDNA was electroeluted in 0.1X TBE buffer (Maniatis et al., 1982, supra). The cDNA was then extracted with phenol and chloroform, and ethanol precipitated (all using procedures of Maniatis et al., 1982, supra) to remove any potential contaminants.

The ethanol-precipitated cDNA was resuspended and was treated with T4 polynucleotide kinase in the presence of ATP in order to add a phosphate molecule to the 5 prime end of each cDNA strand. Seventy-five nanograms of "kinased" cDNA were ligated (using the procedure of Maniatis et al., 1982, supra) together with two micrograms of λgt10 DNA (Stratagene Inc., catalog number GT10), which had previously been digested with the restriction endonuclease EcoRI and dephosphorylated. The λgt10 vector containing cDNA inserts was packaged into infectious phage particles using the "Gigapack Gold" packaging extract obtained from Stratagene, Inc. (catalog number GP10-G). The library was titered using E. coli strains C600 and C600hfl$^+$ (supplied by Stratagene, Inc. with the λgt10 vector) and approximately 1.3 x 10^7 recombinants per μg cDNA were obtained. For amplification of the library, E. coli C600hfl$^+$ cells were infected with the recombinant phage and plated. Ten milliliters of phage storage buffer (10 mM Tris pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$ and 0.15% gelatin) were added to each plate, and the phage were eluted by gently shaking the plates at 4°C overnight. This buffer was then collected and the bacterial cellular debris was pelleted by centrifugation at 8000 rpm for 20 minutes. For prevention of contamination, a small amount of chloroform was added and the amplified library was stored at 4°C.

B. Screening the CHO cDNA Library

The CHO cDNA library was plated (using E. coli strain C600hfl$^+$) at a density of approximately 25,000 plaques per plate. A total of about 150,000 plaques were screened using replica nitrocellulose filters.

Oligonucleotide probes were used to screen the library. For design of the probes, the published sequences of the rat and human facilitative GTPs (Birnbaum et al., Proc. Natl. Acad. Sci. USA, 83:5784-5788, [1986]; Mueckler et al., Science, 229:941-945, [1985]) were compared and three highly conserved sequences were chosen. Single-stranded oligonucleotides to each of these regions were synthesized using standard methodology (Froehler, B. et al., Nucleic Acids Research, 14:5399 [1986]). The sequence of each oligonucleotide is as follows:

GTR-1 (a 75-mer based on the 5' end of human and rat DNA sequences):

5'GCGCAGCGCGGCCATGGAGCCCAGCAGCAAGAAGGTGACGGGC
CGCCTTATGTTGGCCGTGGGAGGGGCAGTGCT

GTR-2 (an 80-mer based on the 3' end of human and rat DNA sequences):

5'AAAGGCCGGACCTTCGATGAGATCGCTTCCGGCTTCCGGCAGGG
GGGTGCCAGCCAGAGCGACAAGACACCTGAGGAGCT

GTR-3 (a 72-mer based on a mid-region section of human and rat DNA sequences):

5'TGTGCAGCAGCCTGTGTATGCCACCATCGGCTCGGGTATCGTCAA
CACGGCCTTCACTGTGGTGTCGCTGTT

Approximately 150,000 plaques on replica nitrocellulose filters from the amplified library were initially screened using the ^{32}P-labeled GTR-2 oligonucleotide in a hybridization solution consisting of 25% formamide, 0.1 mg/ml denatured salmon sperm DNA, 5X SSC (Maniatis et al., 1982, supra), 50 mM sodium phosphate (pH 6.8), 5X Denhardt's (Maniatis et al., 1982, supra) and 20% dextran sulfate at 45°C. One positive plaque was identified after the nitrocellulose filters were washed in 2X SSC and 0.1% SDS at 42°C. The phage from this positive plaque were replated and screened with probes GTR-1 and GTR-3 using the above hybridization and washing conditions, and plaques that hybridized to both of these probes were purified and phage DNA was prepared from one of them. The cDNA inserts were excised from the λgt10 vector by restriction endonuclease digestion with EcoRI, and the size of this cDNA insert was estimated to be approximately 2.5 kb as determined by agarose gel electrophoresis. An agarose gel slice containing this cDNA was cut out and the cDNA was ligated into the pRK5 expression vector described in EP 307,247 published March 15, 1989, which had been previously digested with EcoRI and dephosphorylated (Maniatis et al., 1982, supra) using the method of Struhl, Biotechniques, 3:452-453 [1985]. See Fig. 2. This construct was then transformed into E. coli strain SR101 (which is a strain derived from JM101 [Messing et al., Nucl. Acid. Res., supra] that is λ-resistant, i.e. obtained by selecting for JM101 cells that are not attacked by λ phage) and plated out. Recombinant colonies were picked for sequencing.

C. cDNA Sequencing

Single-stranded DNA template was prepared for sequencing from liquid cultures of the recombinant clones to which helper phage M13K07 (U.S. Biochemical, Inc., catalog #70013) had been added. Two subclones were partially sequenced by the dideoxynucleotide chain termination method (Sanger, F. et al., Proc. Natl. Acad. Sci. USA, 74:5463-5467 [1977]) using the pRK sequencing primer (5'GTGAAATTTGTGATGCT). The sequence obtained from subclone #5 was highly homologous to the 5' end of the rat and human glucose transporter cDNAs, while the sequence from #7 was similar to the 3' end of the rat and human cDNAs and contained a poly(A) tail. The cDNA insert of clone #7 was in the proper orientation in the pRK5 vector for expression in mammalian cells. This plasmid, constructed as shown in Figure 2 and as described above, was named pRK.GTP and was

prepared on a large scale using the alkaline-lysis procedure of Maniatis et al, 1982, supra. Purified plasmid from this preparation was used to transfect CHO cells.

The partial and complete sequences of the CHO cDNA shown in Figures 1 and 11, respectively, were derived from the sequencing gel of pRK.GTP, which was transfected into the cell line GTP-S1 and deposited as ATCC number CRL 10,208. Figure 9 shows the complete nucleotide sequence comparison of rat, CHO, and human GTP. Figure 10 shows the complete amino acid sequence comparison of rat, CHO, and human GTP.

Example 2

Preparation of the Dihydrofolate Reductase (DHFR) Plasmid for Transfection

A full-length cDNA 660-bp fragment encoding murine DHFR was cut from plasmid pDHFR-11 (Chang et al., Nature 275:617 [1978]) with restriction endonucleases Fnu4I and BglII and ligated into the expression vector, pCVESVHBV, which was constructed as follows: The 418 base pair (bp) HpaII-HindIII fragment obtained from plasmid pSVR encompassing the SV40 origin of replication (Liu et al. DNA, 1:213 [1982]) was ligated into plasmid pML (Lusky, M. and Botchan, M., Nature, 293:79 [1981]) between the EcoRI site and the HindIII site. The plasmid EcoRI and the SV40 HpaII sites were made blunt by the addition of Klenow DNA polymerase I in the presence of the four deoxyribonucleotides prior to digestion with HindIII. The resulting plasmid was digested with HindIII and BamHI and the 2900-bp vector fragment isolated. To this fragment was ligated a EcoRI - BamHI fragment of 1985 bp from plasmid pHBV-T-1A (Liu et al., supra) modified to contain a polylinker at the EcoRI site. This fragment encompasses the surface antigen gene (HBsAg). The double-stranded polylinker fragment (5'dAAGCTTATCGATTCTAGAATTC3'...) was digested with HindIII and EcoRI and added to the HBV fragment, converting the EcoRI - BamHI fragment to a HindIII - BamHII fragment. The resulting plasmid, pCVESVHBV, contains a bacterial origin of replication from the pBR322 derived pML, an ampicillin resistance marker (also from pML), an SV40 fragment oriented such that the early promoter will direct the transcription of the ingested HBV fragment, and the HBsAg gene from HBV. The HBV fragment also provides the requisite polyadenylation signal.

For construction of the final plasmid pFD11, the HBsAg coding region of pCVESVHBV was removed by endonuclease digestion of the plasmid with EcoRI. The ends were filled in with Klenow DNA polymerase as described previously, and the vector was then cut with BglII. The Fnu4HI - BglII DHFR cDNA 660-bp fragment from pDHFR-11 was then ligated into the vector, to produce pFD11, as shown in Figure 3.

Example 3

Preparation of t-PA Plasmid for Transfection

Plasmid pRK7 was used as the vector for generation of pRK.t-PA. pRK7 is described in EP 278,776 published August 17, 1988. It is identical to pRK5 (EP Pub. No.

307,247 published March 15, 1989), except that the order of the endonuclease restriction sites in the polylinker region between ClaI and HindIII is reversed. The t-PA cDNA (Pennica et al., Nature, 301:214 [1983]) was prepared for insertion into the vector by cutting with restriction endonuclease HindIII (which cuts 49 base pairs 5' of the ATG start codon) and restriction endonuclease BalI (which cuts 276 base pairs downstream of the TGA stop codon). This cDNA was ligated into pRK7 previously cut with HindIII and SmaI using standard ligation methodology (Maniatis et al., 1982, supra). This construct was named pRK.t-PA.

Example 4

Transfection of CHO Cells

Chinese hamster ovary cell line DUX-B11 (Urlab, G. and Chasin, A.J. Proc. Natl. Acad. Sci. USA, 77:4216-4220 [1980]), which is dihydrofolate reductase minus (DHFR-), was used for all transfections. Cells were maintained in a medium, called PS19, consisting of 1 part of DMEM (ATCC Media Handbook, First Ed., 1984, p. 77), 1 part of Ham's F12 (ATCC Media Handbook, First Ed., 1984, p. 78), and 7.5% FBS. The cells were plated in 60 mm petri plates and grown to about 80% confluence and then transfected using the standard calcium phosphate method (Ausubel et al., eds., Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York, N.Y.). All cells were transfected with 0.5 micrograms per plate of the plasmid pFD11. In addition, all cells except for the control cells were co-transfected with pRK.GTP (at a concentration of 5 micrograms per plate). These cells were designated as GTP-S1 and were deposited with the American Type Culture Collection on August 24, 1989 under the conditions indicated below. Control cells were transfected with 0.5 micrograms of pRK5 per plate.

After 1 - 2 hours, the cells were glycerol shocked and 3 milliliters of PS19 medium (which contains 7.5 percent whole FBS) was added. The cells were grown to confluency and then transferred to a selective medium consisting of PS19 minus glycine, hypoxanthine and thymidine in order to select for cells transfected with and expressing the DHFR gene. Each petri plate culture was then subcultured into several other petri plates, and methotrexate resistance of these subcultures was evaluated over a concentration range of 50nM to 1 μM. Cells resistant to 50nM and 100nM methotrexate were pooled and passaged several times in a medium containing 7.5% FBS. Cell pools were subjected to increasing concentrations of methotrexate during serial passaging, and finally placed in liquid suspension culture in spinner flasks where they were subjected to methotrexate concentrations up to 4 micromolar. The cells were then transferred to a medium containing the commercially available lipid mixture "Ex-cyte" at a concentration of 0.5 percent volume/volume (Miles Laboratories, Inc., catalog number 81-129), trace elements, and transferrin (5 milligrams per liter) but lacking FBS and insulin. After three weeks, some cells were removed from this medium and cultured either in medium supplemented with linoleic acid (1.4 milligrams per liter)

dissolved in ethanol as a lipid source, or in medium lacking lipid. After about three passages, some of the cells were grown in the medium alone (i.e., without trace elements, transferrin, FBS, insulin, linoleic acid, or lipid). Cell viability tests were conducted using Trypan blue dye (which is excluded from the cytoplasm of live cells, which appear colorless, while dead cells cannot exclude the dye and thus appear blue), and cell growth was assessed by counting cells using a hemocytometer.

Figure 5 shows the growth and viability of cells transfected with pFD11 and pRK.GTP grown in medium supplemented with transferrin, trace elements, linoleic acid-ethanol, and insulin as described above. It is apparent that the cell growth rate (Fig. 5A) and viability (Fig. 5B) remained essentially constant over three weeks of culturing.

Figure 6 shows the growth of cells transfected with pFD11 and pRK.GTP and cultured in medium supplemented with transferrin, trace elements, and linoleic acid-ethanol. Very surprisingly, the cell growth (Fig. 6A) and the viability of these cells (Fig. 6B) did not decrease at all (in contrast to the control), even though neither serum nor insulin was added to the culture medium. This suggests that the increased number of GTP molecules on the surface of the plasma membrane of these cells enables the cells to overcome the requirement for insulin and/or FBS in the culture medium.

The data in Figure 7 show cells grown in the same medium as in Figure 6 but without linoleic acid-ethanol. Clearly, the cell growth (Fig. 7A) and cell viability (Fig. 7B) are maintained over several passages. Further, linoleic acid-ethanol does not appear to enhance growth and survival of the cells, as growth and viability of these cells is essentially equivalent to that in Figure 6. Control cells containing amplified pRK5 but not pRK.GTP are incapable of survival in this medium.

Figure 8 shows the growth (Fig. 8A) and viability (Fig. 8B) of cells transfected with pFD11 and pRK.GTP cultured in medium without any supplements (i.e., without trace elements, serum, transferrin, linoleic acid, or lipid). The results, which indicate that normal cell growth and viability were observed, are most unexpected; it is highly unusual for eukaryotic cells to survive several passages in medium lacking growth factors, a lipid source, and insulin. Control cells not containing pRK.GTP, but transfected with pRK5, are incapable of survival in this medium.

The transfection procedure described above was repeated except that in addition to transfection with pFD11 and pRK.GTP or pFD11 and pRK5 (control), cells were also transfected with pRK.t-PA. It is expected that the growth and viability of the cells transfected with pFD11, pRK.GTP, and pRK.t-PA would not be adversely affected if neither serum, linoleic acid-ethanol, transferrin, nor insulin were added to the medium. Growth and survival of the cells transfected with pRK.t-PA alone, however, would be expected to be adversely affected by the lack of such additives.

Example 5

Southern Blot Analysis

Southern blot analysis was conducted essentially as described in sections 9.31-9.58 of Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York [1989]).

Genomic DNA was isolated from Chinese hamster ovary cells which had been previously transfected with pRK.GTP as described above. The DNA was extracted and purified using a kit and the accompanying protocol purchased from the Oncor Corporation. Purified DNA was digested with restriction endonuclease EcoR1, BamH1, or both enzymes using standard procedures. The digested DNA was electrophoresed through an agarose gel and then transferred on to a nylon membrane (GeneScreen, purchased from DuPont). Transfer of the DNA to the nylon membrane was accomplished by the standard capillary transfer method (sections 9.32-9.43 of Sambrook et al., supra) using an alkaline buffer (for example, 0.04 M NaOH, 0.06 M NaCl).

After transfer overnight, the membrane was baked for 2 hours under vacuum at 80°C to immobilize the DNA and then prehybridized at 43°C for two hours with 0.5 mg/ml E. coli DNA, 50% formamide, 5X SSPE (Sambrook et al., supra, section B.13) and 5X Denhardt's solution (Sambrook et al., supra, section 9.49).

To prepare the probe, plasmid pRK.GTP was radio-labelled with 32-P dCTP using the following method. Two micrograms of DNA were added to 5 µl of nick-translation buffer (obtained from New England Nuclear), 6µl water, and 1 µl of a solution containing 2 mM dATP, 2mM dTTP, and 2 mM dGTP. To this was added 5 µl of a stock solution of 32-P dCTP with a specific activity of 3000Ci/mmol (10 mCi/ml). The nick-translation reaction was started by the addition of 2 µl of DNA polymerase I and 2 µl of DNAse I. This solution was incubated at 15°C for 1 hour, and then terminated by the addition of 20 µl of stop buffer (such as 0.5 M EDTA at pH 8.0). The DNA was then purified using the ammonium acetate/ethanol precipitation method (section 6.34 of Sambrook et al., supra) and then resuspended in 100 µl of water, and 100 µl of formamide was added. After the DNA solution was heated to 100°C, it was added to the Southern blot and hybridized at 42°C overnight. After hybridization, the blot was washed at 65°C for 15 minutes in 30 ml (although 300 ml would also be acceptable) of 2X SSC (Sambrook et al., supra, section B.13) and 0.5% SDS. This wash step was repeated twice. The blot was then allowed to dry before sealing it in plastic wrap and exposing it to film.

The results of the Southern blot analysis show that the probe hybridized to a particular band of DNA only in the cells transfected with pRK.GTP. The probe did not hybridize to a corresponding band in the genomic DNA of the control cells (which had been transfected with only the pRK vector).

Deposit of Materials

The following culture has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

Strain	ATCC Accession No.	Deposit Date
GTP-S1	CRL 10,208	August 24, 1989

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from the date of deposit. The organism will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if the culture on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustration that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method comprising transfecting eukaryotic cells with a replicable vector comprising a nucleic acid sequence encoding a GTP operably linked to control sequences recognized by the transfected cells.
2. The method of claim 1 wherein the eukaryotic cells are mammalian cells.
3. The method of claim 2 wherein the mammalian cells are Chinese hamster ovary cells.
4. The method of claim 1 wherein the eukaryotic cells are insect cells.
5. The method of claim 1 wherein the eukaryotic cells are also transformed with the vector.
6. The method of claim 1 wherein the cells additionally are cotransfected with a vector comprising a nucleic acid sequence encoding a desired polypeptide operably linked to control sequences recognized by the transfected cells.
7. The method of claim 6 wherein the desired polypeptide is a heterologous polypeptide.
8. The method of claim 6 wherein the desired polypeptide comprises transforming growth factor-beta, CD-4, transforming growth factor-alpha, activin, tissue plasminogen activator, DNase, insulin-like growth factor, or nerve growth factor.
9. The method of claim 1 wherein the cells prior to transfection with the GTP require serum for growth or survival, but do not require serum for growth or survival after transfection.
10. The method of claim 1 wherein the cells prior to transfection with the GTP require linoleic acid for survival or growth, but do not require linoleic acid after transfection.
11. The method of claim 1 wherein the cells are additionally cotransfected with a vector comprising a nucleic acid sequence encoding a selectable marker operably linked to control sequences recognized by the transfected cells.
12. The method of claim 11 wherein the selectable marker is dihydrofolate reductase (DHFR) and the selection agent is an inhibitor of DHFR or a selection medium lacking glycine, hypoxanthine, and thymidine.
13. The method of claim 12 wherein the selection agent is methotrexate.
14. The method of claim 7 wherein the cells additionally are cotransfected with a vector comprising a nucleic acid sequence encoding a selectable marker operably linked to control sequences recognized by the transfected cells.
15. The method of claim 13 wherein the selectable marker is dihydrofolate reductase (DHFR) and the selection agent is an inhibitor of DHFR or a selection medium lacking glycine, hypoxanthine, and thymidine.
16. The method of claim 15 wherein the selection agent is methotrexate.
17. The method of claim 1 wherein the vector is an expression vector.

18. The method of claim 17 wherein the expression vector further comprises a nucleic acid sequence encoding a desired polypeptide operably linked to control sequences recognized by the transfected cells.
19. The method of claim 18 wherein the desired polypeptide is a heterologous polypeptide.
20. The method of claim 18 wherein the polypeptide is not a selectable marker.
21. The method of claim 18 wherein the desired polypeptide comprises transforming growth factor-beta, CD4, transforming growth factor-alpha, activin, tissue plasminogen activator, DNase, insulin-like growth factor, or nerve growth factor.
22. The method of claim 1 wherein the GTP is homologous to the eukaryotic cells.
23. The method of claim 3 wherein the GTP is homologous to the Chinese hamster ovary cells.
24. The method of claim 1 wherein the GTP is heterologous to the eukaryotic cells.
25. The method of claim 1 wherein the nucleic acid sequence is a DNA sequence.
26. A host cell produced by the method of claim 1.
27. An isolated nucleic acid sequence encoding a GTP, which sequence comprises at its 5' end the nucleotide sequence shown in Fig. 1, where the start ATG codon is indicated.
28. The sequence of claim 27 that is a DNA sequence and that further comprises the sequence upstream of the start codon in Figure 9A under the "CHO" name.
29. The nucleic acid sequence of claim 27 further comprising a promoter operably linked to said nucleic acid sequence.
30. An expression vector comprising the nucleic acid sequence of claim 29 operably linked to control sequences recognized by a host transformed by the vector.
31. A host cell transformed with the expresion vector of claim 30.
32. The host cell of claim 31 wherein the cell is eukaryotic.
33. A eukaryotic cell comprising a replicable vector comprising a nucleic acid sequence encoding a GTP operably linked to control sequences recognized by the eukaryotic cells.
34. The cell of claim 33 that is a mammalian cell, wherein the GTP is a Chinese hamster, rat, rabbit, chick, or human protein.
35. The cell of claim 34 that is a Chinese hamster ovary cell.
36. The cell of claim 33 that is an insect cell.
37. The cell of claim 33 wherein the replicable vector is an expression vector further comprising a nucleic acid sequence encoding a desired polypeptide operably linked to control sequences recognized by the eukaryotic cells.

38. The cell of claim 33 further comprising an expression vector comprising a nucleic acid sequence encoding a desired polypeptide operably linked to control sequences recognized by the eukaryotic cell.
39. The cell of claim 33 further comprising an expression vector comprising a nucleic acid sequence encoding a selectable marker operably linked to control sequences recognized by the eukaryotic cell.
40. The cell of claim 38 further comprising an expression vector comprising a nucleic acid sequence encoding a selectable marker operably linked to control sequences recognized by the eukaryotic cell.
41. The cell of claim 39 wherein the selectable marker is dihydrofolate reductase.
42. The cell of claim 40 wherein the selectable marker is dihydrofolate reductase.
43. The cell of claim 33 wherein the GTP is homologous to the eukaryotic cell.
44. The cell of claim 33 wherein the GTP is heterologous to the eukaryotic cell.
45. The cell of claim 33 wherein the nucleic acid sequence is a DNA sequence.
46. The cell of claim 33 that does not require serum for serial culture.
47. The cell of claim 33 that does not require linoleic acid for serial culture.
48. The cell of claim 33 that does not require insulin for serial culture.
49. The cell of claim 33 wherein the cell prior to transfection with the vector requires serum for survival or growth.
50. An isolated eukaryotic cell having its origin in a multicellular organism, which cell constitutively expresses GTP so that the cell is not dependent upon serum, insulin, or transferrin for growth or survival.
51. The host cell of claim 31 with ATCC number CRL 10,208.
52. An isolated DNA sequence encoding a glucose tarnsporter protein, which sequence comprises the nucleotide sequence shown in Figure 1 from the start to the stop codons.

Fig.1.

```
   |START|
5'-A T G G A G C C C A G C A C C A A G A A C G T G A C T G G C C C C T C
3'-T A C C T C G G G T C G T G G T T C T T G C A C T G A C C G G G G A G
   Met   Glu   Pro   Ser   Ser   Lys   Lys   Val   Thr   Gly   Arg   Leu

   A T G C T G G C C C G T G G G A G G G G C A G T G C T C G G A T C C C T G
   T A C G A C C G G G C A C C C T C C C C G T C A C G A G C C T A G G G A C
   Met   Leu   Ala   Val   Gly   Gly   Ala   Val   Leu   Gly   Ser   Leu

   C A G T T T G G C T A T A A C A C T G G C G T C A T C A A T G C C C C C
   G T C A A A C C G A T A T T G T G A C C G C A G T A G T T A C G G G G G
   Gln   Phe   Gly   Tyr   Asn   Thr   Gly   Val   Ile   Asn   Ala   Pro

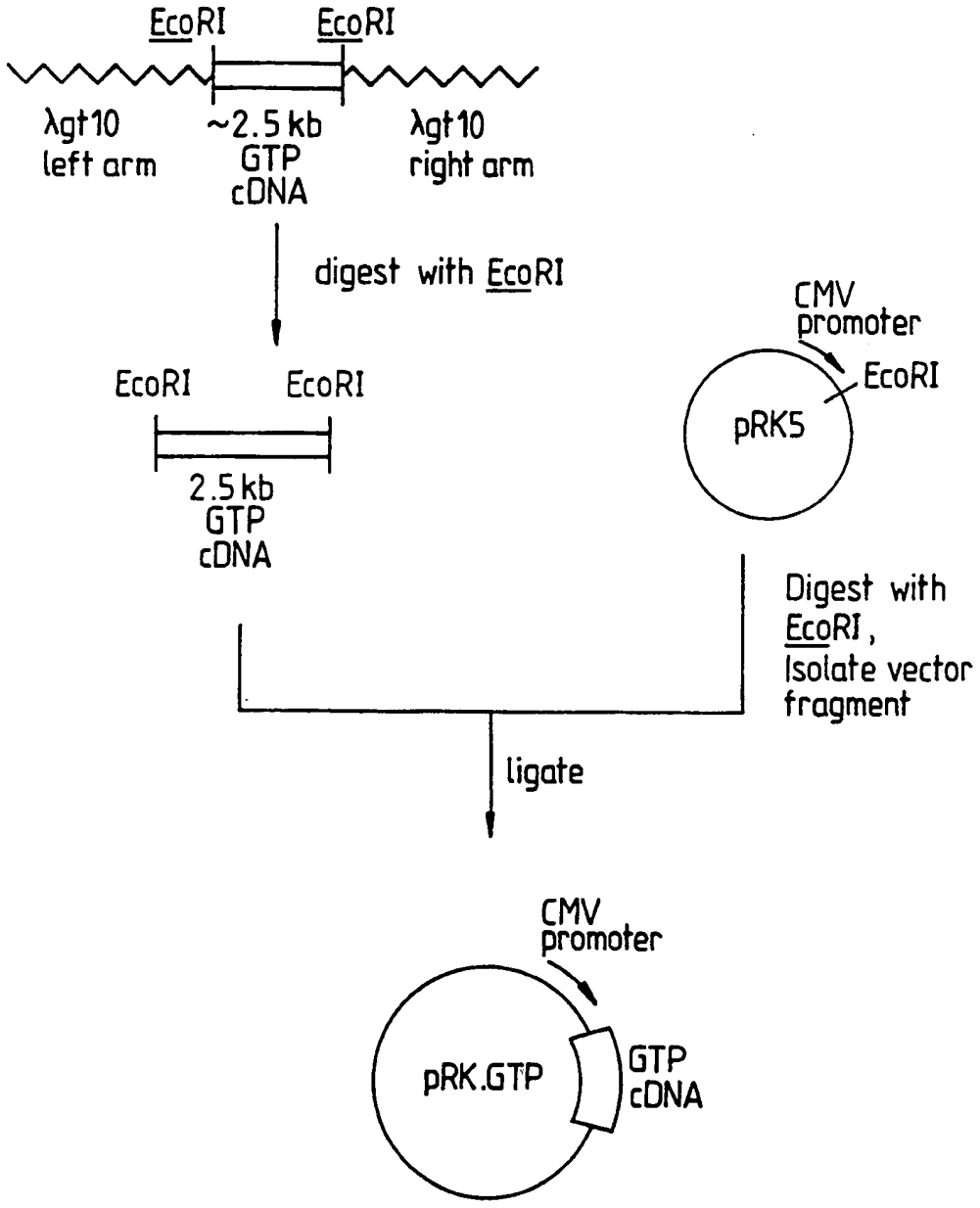
   C A G A A G G T A A T T G A G G A G T T C T A C A A C C A G A C A T G G
   G T C T T C C A T T A A C T C C T C A A G A T G T T G G T C T G T A C C
   Gln   Lys   Val   Ile   Glu   Glu   Phe   Tyr   Asn   Gln   Thr   Trp

   G T C C A C C G C T A T G G A G A G C C C A T T G T G C C C A C C A C A A
   C A G G T G G C G A T A C C T C T C G G G T A A C A C G G G T G G T G T T
   Val   His   Arg   Tyr   Gly   Glu   Pro   Ile   Val   Pro   Thr   Thr

   C T C A C C A C G C T C T G G T C C C T C T C G G T G G C C A T C T T C
   G A G T G G T G C G A G A C C A G G G A G A G C C A C C G G T A G A A G
   Leu   Thr   Thr   Leu   Trp   Ser   Leu   Ser   Val   Ala   Ile   Phe

   T C C G T C G G G G G G -3'
   A G G C A G C C C C C C -5'
   Ser   Val   Gly   Gly
```

Fig.2.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EcoRI
λgt10 left arm
~2.5 kb GTP cDNA
λgt10 right arm
digest with EcoRI
EcoRI
EcoRI
2.5 kb GTP cDNA
CMV promoter
EcoRI
pRK5
Digest with EcoRI, Isolate vector fragment
ligate
CMV promoter
pRK.GTP
GTP cDNA

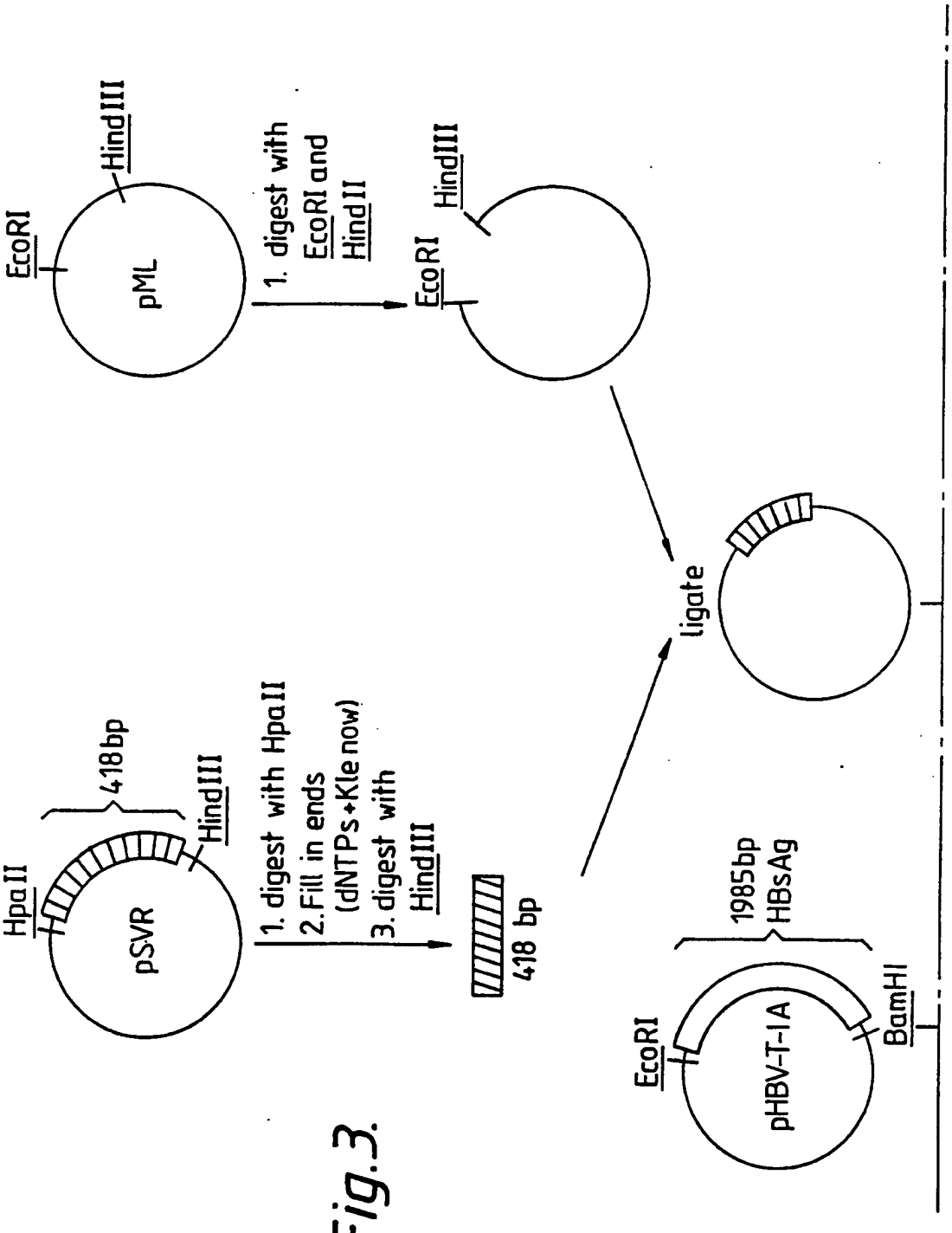

EcoRI
HindIII
pML
1. digest with
EcoRI and
HindII
EcoRI
HindIII
ligate
HpaII
418bp
HindIII
pSVR
1. digest with HpaII
2. Fill in ends
(dNTPs+Kle now)
3. digest with
HindIII
418 bp
1985bp
HBsAg
EcoRI
pHBV-T-1A
BamHI

Fig.3.

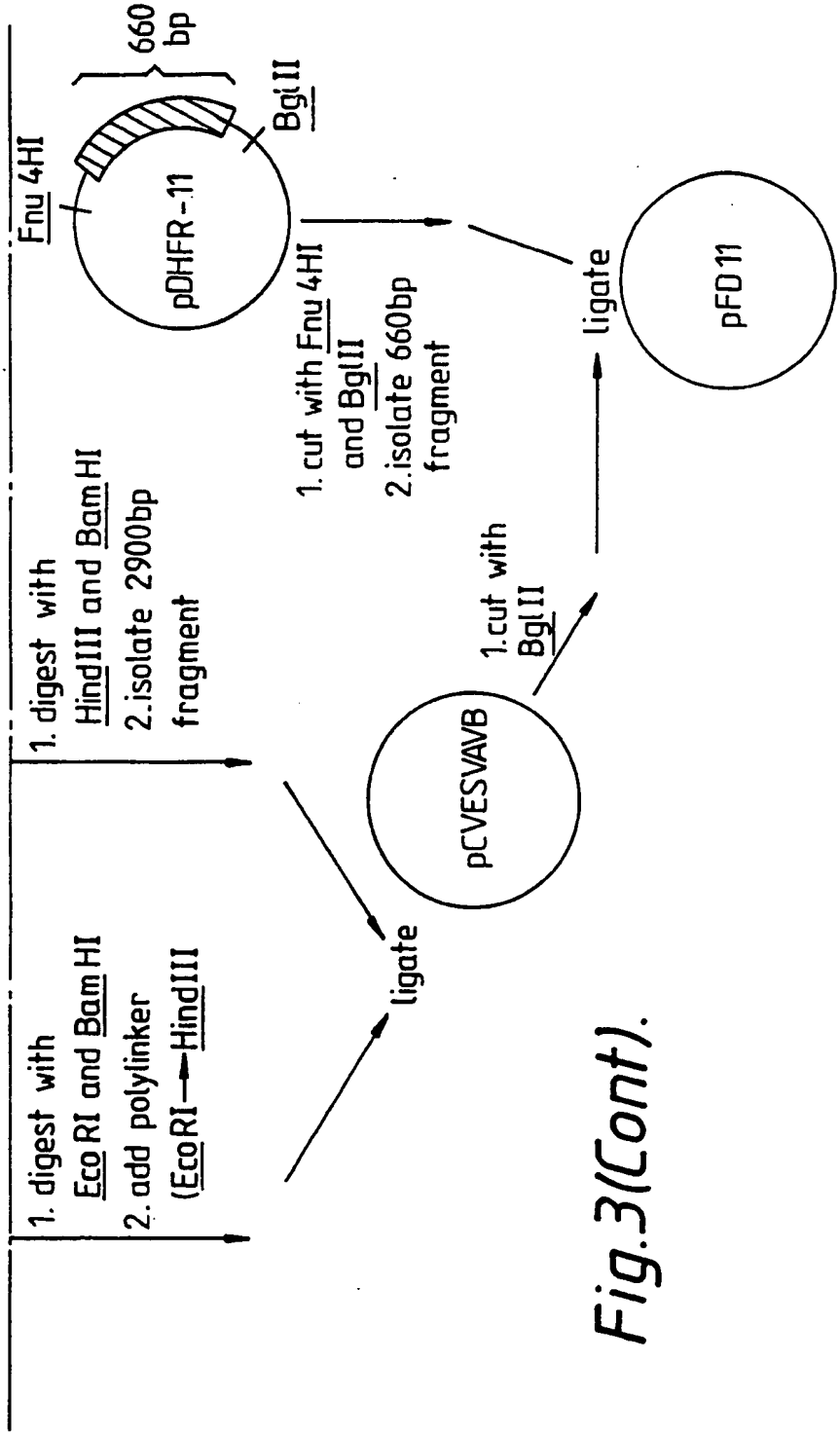

1. digest with
EcoRI and BamHI
2. add polylinker
(EcoRI→HindIII
1. digest with
HindIII and BamHI
2. isolate 2900bp
fragment
ligate
pCVESVAVB
1. cut with
BglII
ligate
pFD11
1. cut with FnuHI
and BglII
2. isolate 660bp
fragment
pDHFR-11
FnuHI
BglII
660
bp

Fig.3(Cont).

Fig.4.

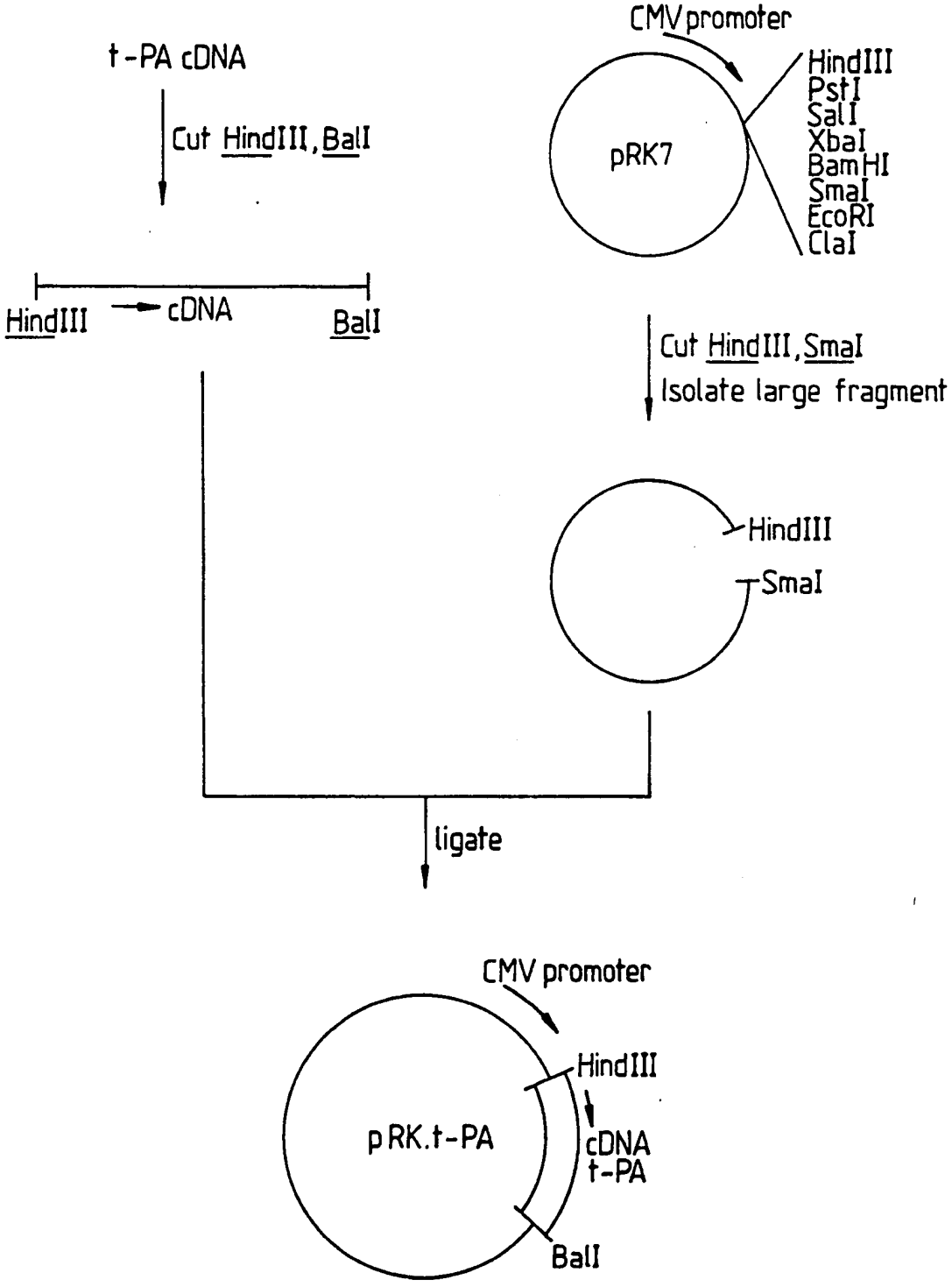

t-PA cDNA
Cut HindIII, BalI
HindIII
cDNA
BalI
CMV promoter
pRK7
HindIII
PstI
SalI
XbaI
BamHI
SmaI
EcoRI
ClaI
Cut HindIII, SmaI
Isolate large fragment
HindIII
SmaI
ligate
CMV promoter
pRK.t-PA
HindIII
cDNA
t-PA
BalI

Fig.5A.

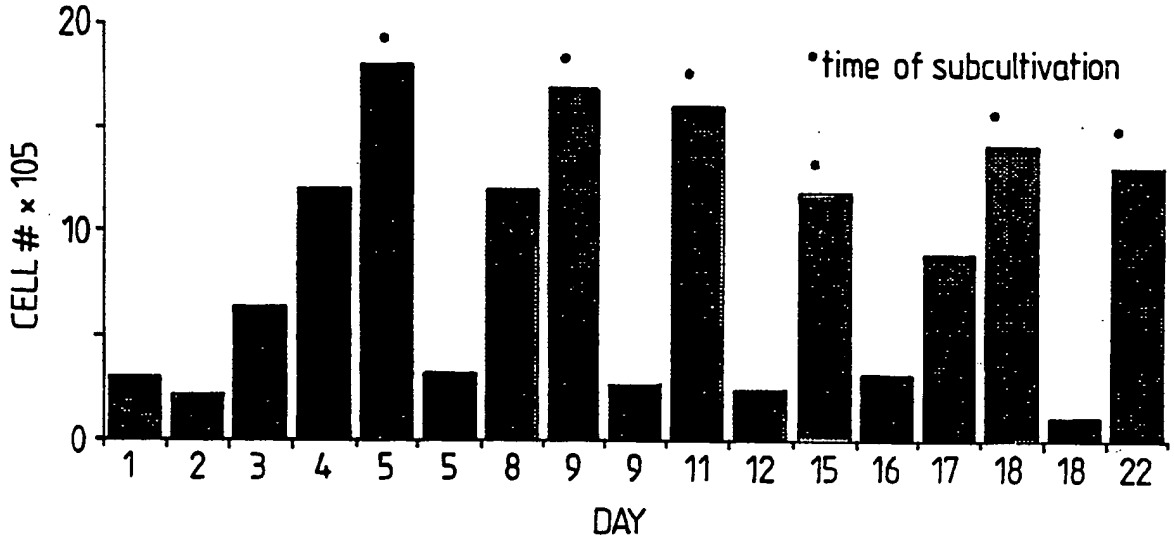

20
10
0
CELL # x 105
•time of subcultivation
1 2 3 4 5 5 8 9 9 11 12 15 16 17 18 18 22
DAY

Fig.5B.

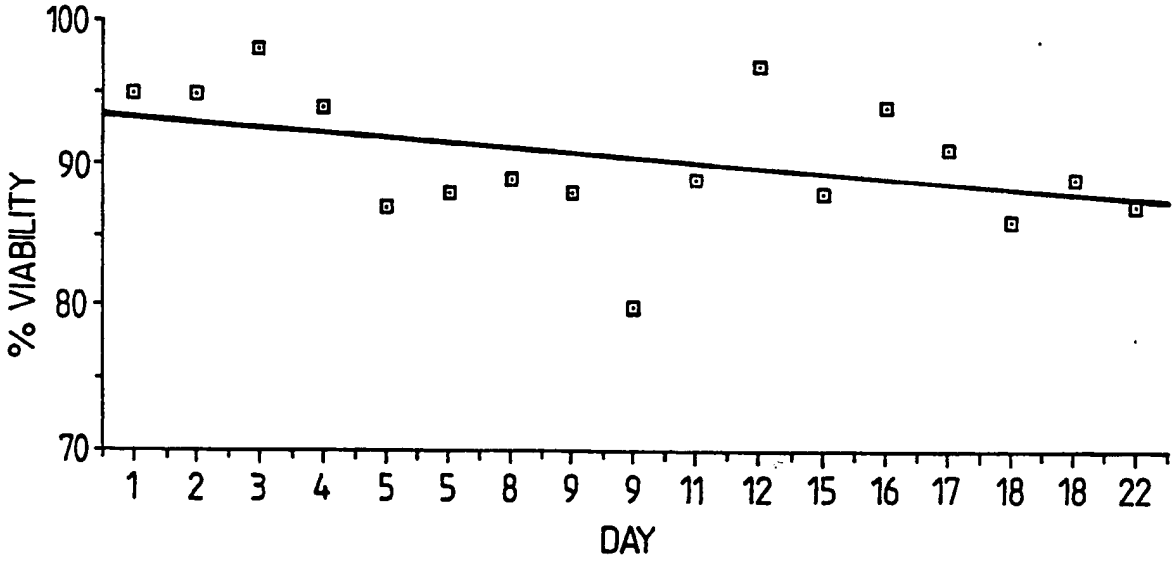

100
90
80
70
% VIABILITY
1 2 3 4 5 5 8 9 9 11 12 15 16 17 18 18 22
DAY

Fig.6A.

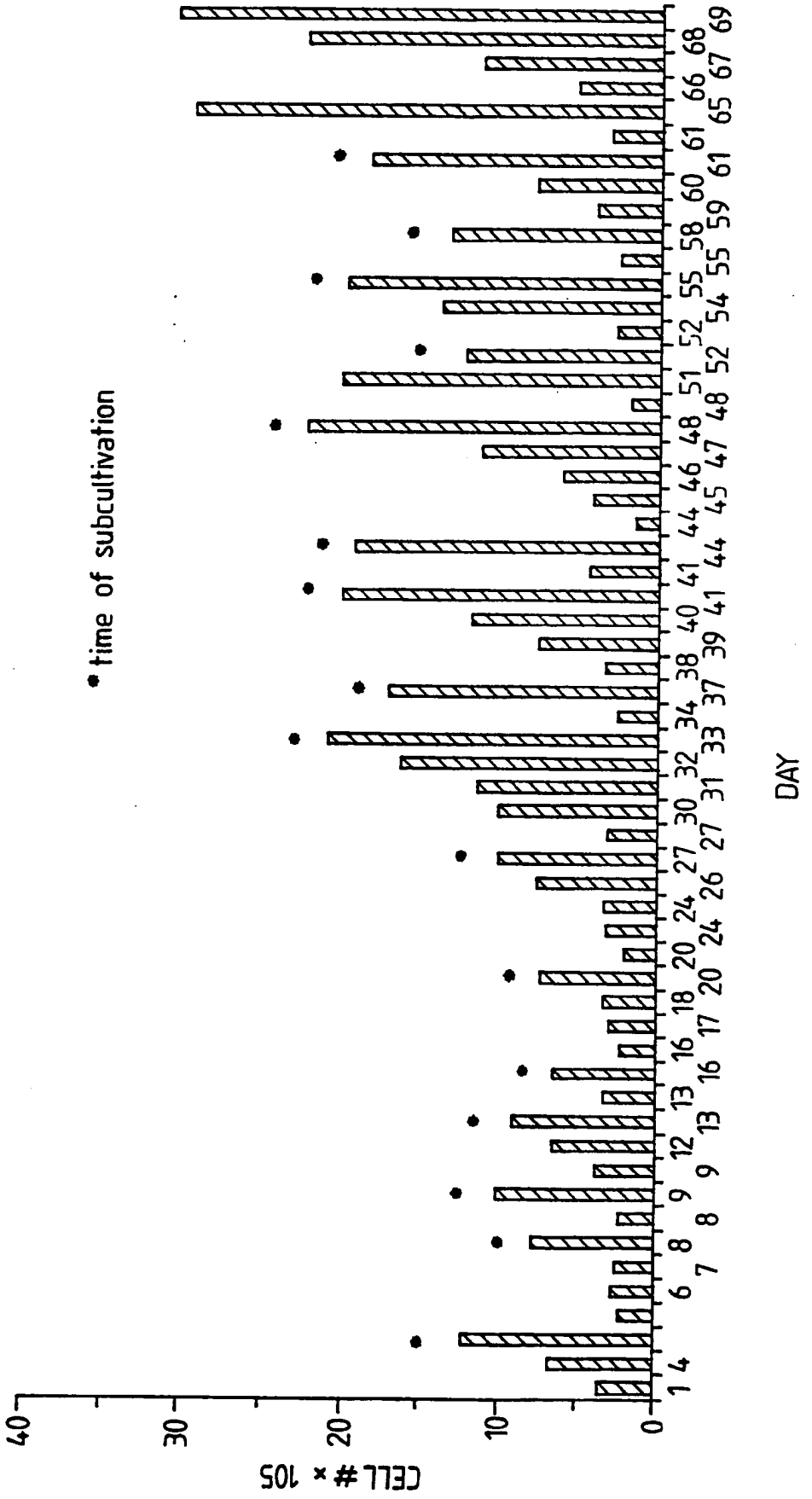

• time of subcultivation
CELL # x 10⁵
0
10
20
30
40
DAY
1 4 6 7 8 8 9 9 12 13 13 16 16 17 18 20 20 24 24 26 27 27 30 31 32 33 34 37 38 39 40 41 41 44 44 45 46 47 48 48 51 52 52 54 55 55 58 59 60 61 61 65 66 67 68 69

Fig.6B.

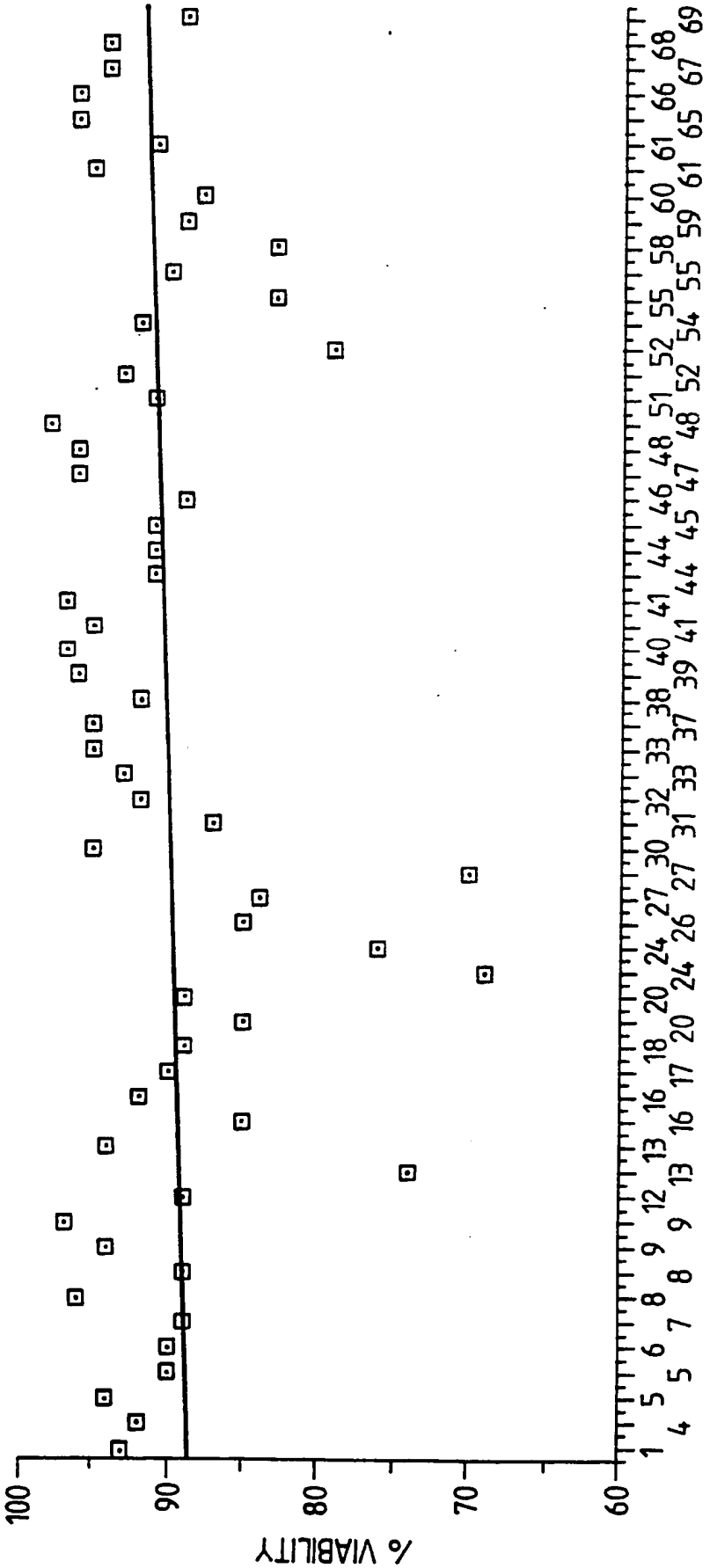
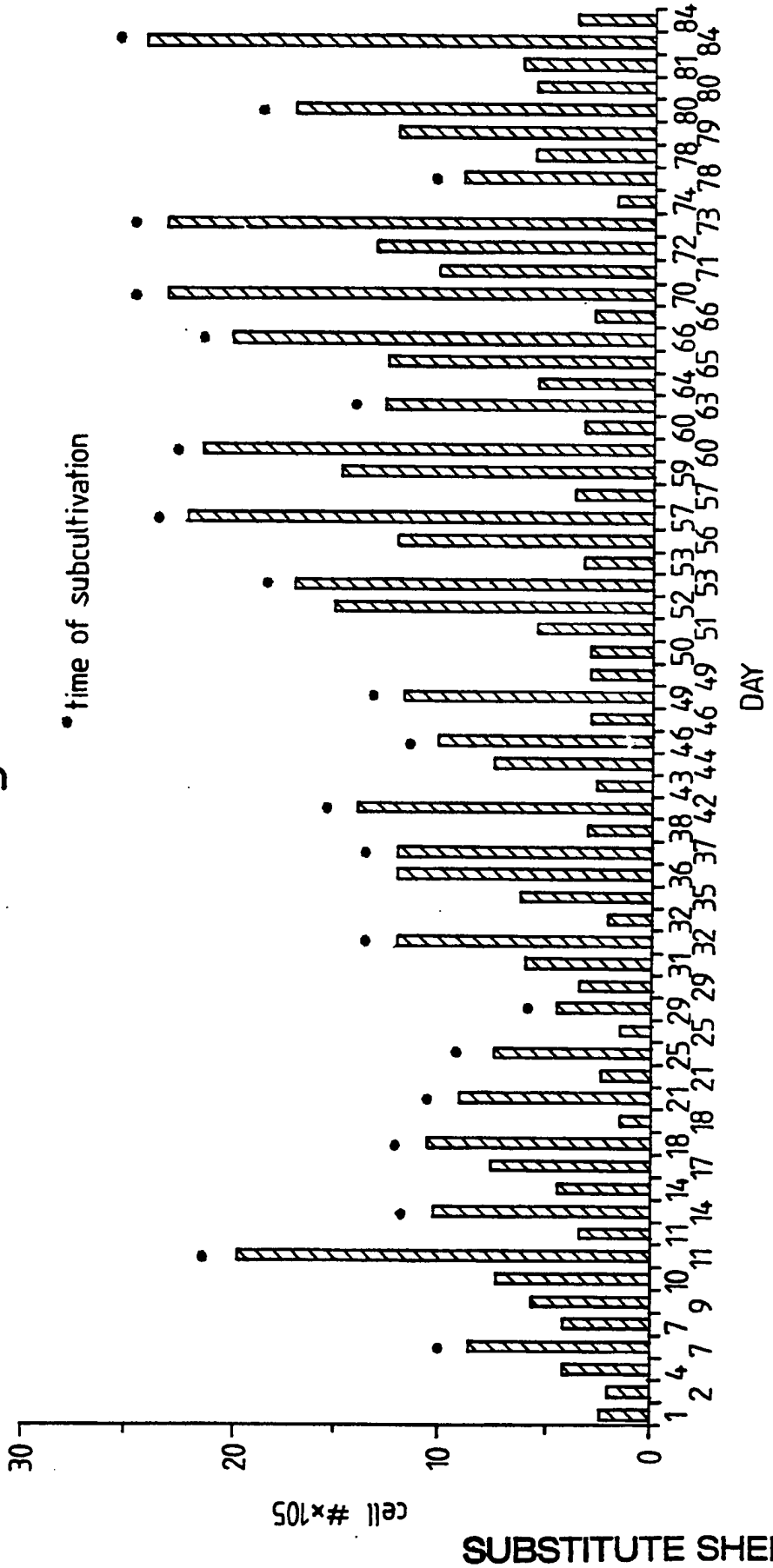

% VIABILITY
100
90
80
70
60
DAY
1 4 5 6 7 8 9 12 13 16 16 17 18 20 24 24 26 27 27 30 31 33 33 37 38 39 40 41 44 44 45 46 47 48 48 51 52 54 55 55 58 59 61 60 61 65 66 67 68 69

Fig. 7A.


cell #x10⁵
30
20
10
0
DAY
1 2 4 7 7 9 10 11 11 14 14 17 18 18 21 21 25 25 29 29 31 32 32 35 36 37 38 42 43 44 46 46 49 49 50 51 52 53 53 56 57 57 59 60 60 63 64 65 66 66 70 71 72 73 74 78 78 79 80 80 81 84 84
time of subcultivation

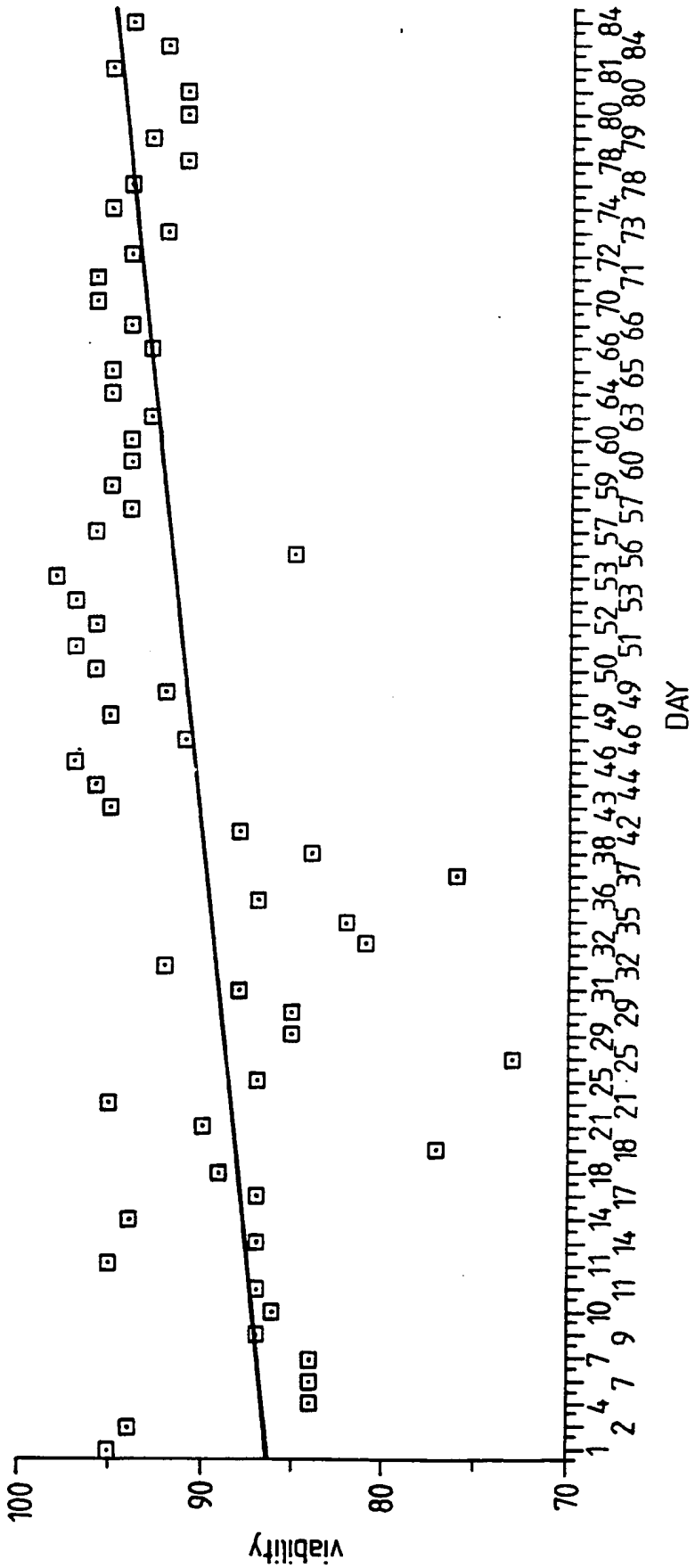
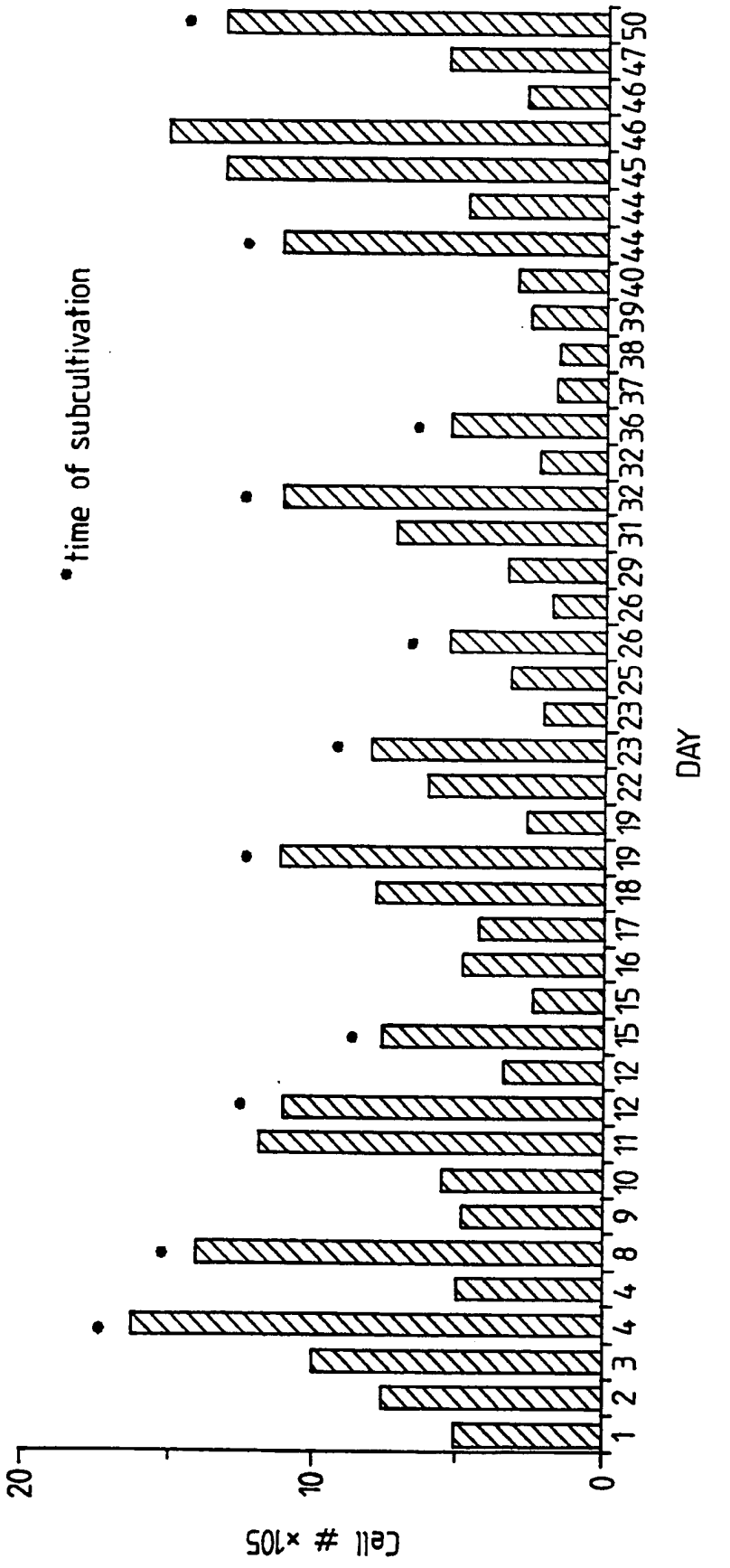

viability
100
90
80
70
DAY
1 2 4 7 7 9 10 11 11 14 14 17 18 18 21 21 25 25 29 29 31 32 32 35 36 37 38 42 43 44 46 46 49 49 50 51 52 53 53 56 57 57 59 60 60 63 64 65 66 66 70 71 72 73 74 78 78 79 80 80 81 84 84

Fig.7B.

Fig.8A.


time of subcultivation
Cell # x10⁵
20
10
0
1 2 3 4 4 8 9 10 11 12 12 15 15 16 17 18 19 19 22 23 23 25 26 26 29 31 32 32 36 37 38 39 40 44 44 45 46 46 47 50
DAY

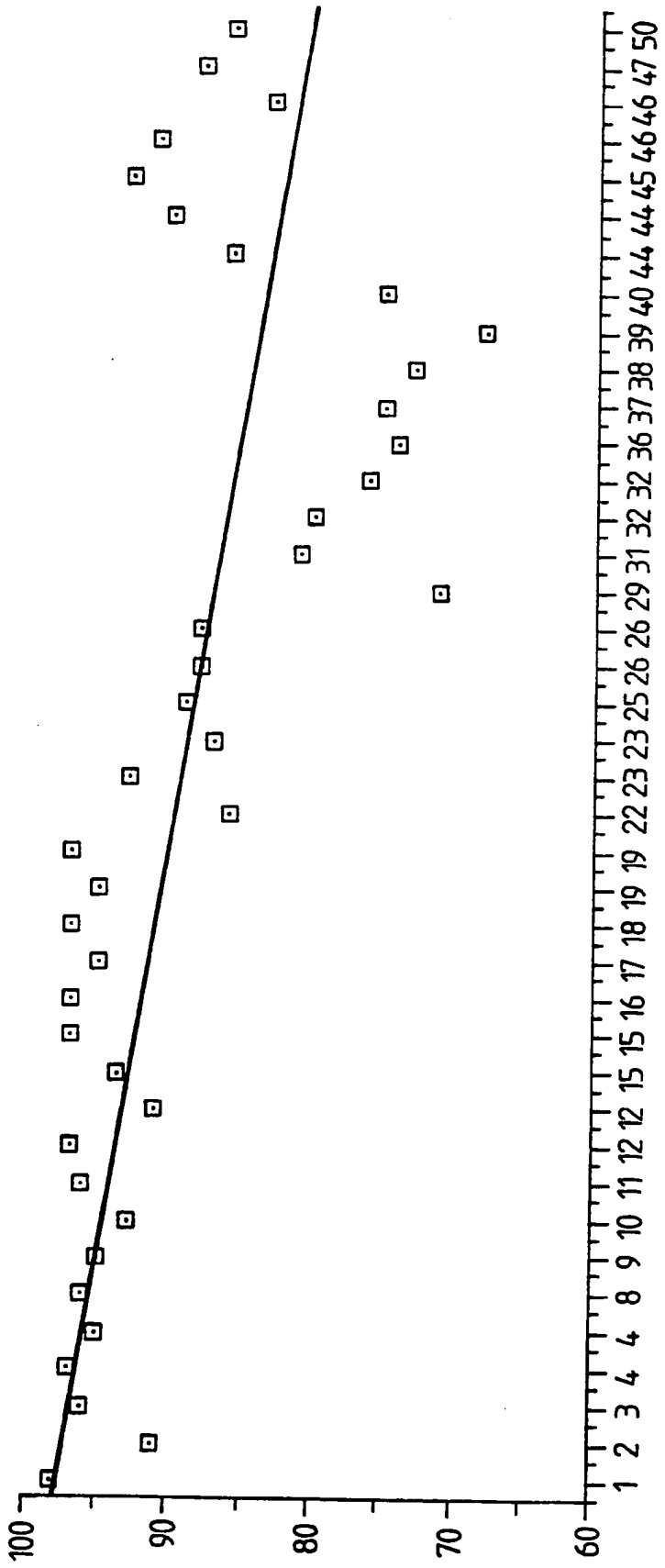

100
90
80
70
60
Viability
1 2 3 4 4 8 9 10 11 12 12 15 15 16 17 18 19 19 22 23 23 25 26 26 29 31 32 32 36 37 38 39 40 44 44 45 46 46 47 50
DAY

Fig.8B.

Fig. 9A.

```
cho  ----------GGCGGTCCTATAAAAGGCTCTCACGGCGCGCT
rat  GGGGCGGGCCAATGGCGGCGGTCCTATAAAAGGCACGCTCCGCGCTCT
hum  ----------TAGTCGCGGGTCCCCGAGTGA

cho  CTCCGCACAGCTCAGAATTACTCGTGAGAGTGCTGGTCTAGGCTACGGG
rat  CTTCCTAAGAACACAAGAATCCCTTGTGGAGTGTCGGTTTAGGTTGCAGG
hum  GCACGCCAGGAGCAGGAACCAACGAGGGGGTCGGAGTCAGAGTCGCC

cho  GTCCCAAGAGTGCCAAGGCGTGGAGAGATCCGGCGGGAGAGCAGTCAGA
rat  GTCTTAAGTGAGTCAGGGCGCGGAGGGTCCGGCGGAGAGCATAGTCACA
hum  AGTGGGAGTCCCCGGAACCGGACACGAGCCTGAGGCGGGAAGGCCGCTC

cho  GCACGCCCCGTTCCCTGTCTCGCAGCCAGCTAGAGGCACGATCGCA
rat  GAACGTCCTTCTCCTGTTCACAGCCCGCACACAGCTTGAGCCTCGAGCGCA
hum  GCACGCCCCGTCGCCACCCGCGTACCCCGGCGCAGCCACAGAGCCACGCCA

cho  GCGCGGCCATGGAGCCCAGCAAGAAGAGGTGACTGGCCGCCTCATGCTG
rat  GCGCGGCCATGGAGCCCAGCAAGAAGAGGTGACGGGCCGCCTTATGTTG
hum  GCGCTGCCATGGAGCCCAGCAAGAAGCTGACGGGTCGCCTCATGCTG
                    Start!

cho  GCCGTGGGAGGGGCAGTGCTCGGATCCCCTGCAGTTTGGCTATAACACTGG
rat  GCCGTGGGAGGGGCAGTGCTCGGATCCCCTGCAGTTCGGCTATAACACCGG
hum  GCTGTGGGAGGAGCAGTGCTTGGCTCCCCTGCAGTTTGGCTACAACACTGG

cho  CGTCATCAATGCCCCCCAGAAGGTAATTGAGGAGTTCTACAACCAGACAT
rat  TGTCATCAACGCCCCCCAGAAGGTAATTGAGGAGTTCTACAACAATCACAT
hum  AGTCATCAATGCCCCCCAGAAGGTGATCGAGGAGTTCTACAACCAGACAT
```

Fig. 9B.

```
cho GGGTCCACCGCTATGGAGAGCCCATTGTGCCCACCACACTCACCACGCTC
rat GGAACCACCGCTATGGAGAGTCCATCCCACCACACTCACCACACTC
hum GGGTCCACCGCTATGGGAGAGCATCCTGCCCACCACACGCTCGCTCACCACGCTC

cho TGGTCCCTCTCTCGGTGGCCATCTTCTCCGTCGGGGGCATGATTGGTTCCTT
rat TGGTCTCTCTCCGTGGCCATCTTCTGTCGGGGGCATGATTGGTTCCTT
hum TGGTCCCTCTCAGTGGCCATCTTTTCTGGGGGCATGATTGGCTCCTT

cho TCTGTGGGCCTCTTTGTTAATCGCTTTGGCAGGCGGAACTCCATGCTGA
rat CTCTGTGGGCCTCTTTGTTAATCGCTTTGGCAGGCGGAACTCCATGCTGA
hum CTCTGTGGGCCTTTGTTAACGCTTTGGCGGCGGAATTCAATGCTGA

cho TGATGAACCTATTGGCCTTTGTGTCTGCTGTGCTCATGGGCTTCTCCAAA
rat TGATGAACCTGTTGGCCTTTGTGTCTGCCGTGCTTATGGGTTCTCCAAA
hum TGATGAACCTGCTGGCCTTCGTGTCGCCGTGCTCATGGGCTTCTCGAAA

cho CTGGGCAAGTCTTTGAGATGCTGATCCTGGGCCGCTTCATCATTGGAGT
rat CTGGGCAAGTCCTTTGAGATGCTGATCCTGGGCCGCTTCATCATTGGAGT
hum CTGGGCAAGTCCTTTGAGATGCTGATCCTGGGCCGCTTCATCATCGGTGT

cho GTACTGTGGCCTGACCACTGGCTTTGTGCCCATGTATGTGGGAGAGGTGT
rat GTACTGTGGCCTGACCACCGGCTTTGTGCCCATGTATGTGGGGGAGGTGT
hum GTACTGCGGCCTGACCACAGGCTTCGTGCCCATGTATGTGGGTGAATGTGT

cho CACCCACAGCTCTTCGTGGAGCCCTAGGCACGCTGCTGCACCAGCTGGGCATC
rat CACCCACAGCTCTTCGTGGAGCCCCTGGGCACCCTGCACCAGCTGGGCATC
hum CACCCACAGCCTTTCGTGGGCCCCTGGGCACCCTGCACCAGCTGGGCATC
```

Fig. 9C.

```
cho GTCGTTGGCATCCTTATTGCCCAGGTGTTCGGCCTGGACTCCATTATGGG
rat GTCGTTGGATCCTTATTGCCCAGGTGTTCGGCTTAGACTCCATCATGGG
hum GTCGTCGGCATCCTCATCGCCCAGGTGTTCGGCCTGGACTCCATCATGGG

cho CAATGCAGACTTGTGGCCTCTGCTGCTTCAGTATCATCTTCGTCCCAGCCC
rat CAATGCAGACTTGTGGCCTCTACTGCTTCAGTGTCATCTTCATCCCAGCCC
hum CAACAAGGACCTGTGGCCCCTGCTGCTTGAGCATCATCTTCATCCCGGCCC

cho TGCTACAGTGCATCCTGTTGCCCCTTCTGCCCCGAGAGCCCCCCGCTTCCTG
rat TGCTACAGTGTATCCTGTTGCCCCTTCTGCCCCTGAGAGCCCCCCGCTTCCTG
hum TGCTGGCAGTGCATCGTGCTGCCCCTTCTGCCCCCGAGAGTCCCCGCTTCCTG

cho CTCATCAATCGCAACGAGGAAACCGGGGCCAAGAGTGTGCTGAAGAAGCT
rat CTCATCAATCGTACGAGGAGAACCGGGCCAAGAGTGTGCTGAAACAAGCT
hum CTCATCAACCGCAACGAGGAGAACCGGGGCCAAGAGTGTGCTAAGAAGCT

cho TCGAGGGGACAGCCGATGTGACCCCGAGACCTGCAGGAGATGAAAGAAGAGG
rat TCGAGGGGACAGCCGATGTGACCCCGAGACCTGCAGGAGATGAAAGAAGAGG
hum GCGCGGGACAGCTGAGTGACCCATGACCTGCAGGAGATGAAGGAGAGGA

cho GTCGGCAGATGATGAGGAGAAGAGGTCACCATCCCTGGAGCTGTTCCGC
rat GTCGGCAGATGATGAGGAGAAGAGGTCACCATCCTTGGAGCTGTTCCGC
hum GTCGGCAGATGATGCGGGAGAAGAGGTCACCATCCTGGAGCTGTTCCGC

cho TCAGCCGCCTACCGCCAGCCCATCCTCATTGCTGTGGTGCTGCAGCTGTC
rat TCACCCGCCTACCGCCAGCCCATCCTCATCGCCGTGGTGCTGCAGCTGTC
hum TCCCCGCCTACCGCCAGCCCATCCTCATCGCTGTGGTGCTGCAGCTGTC
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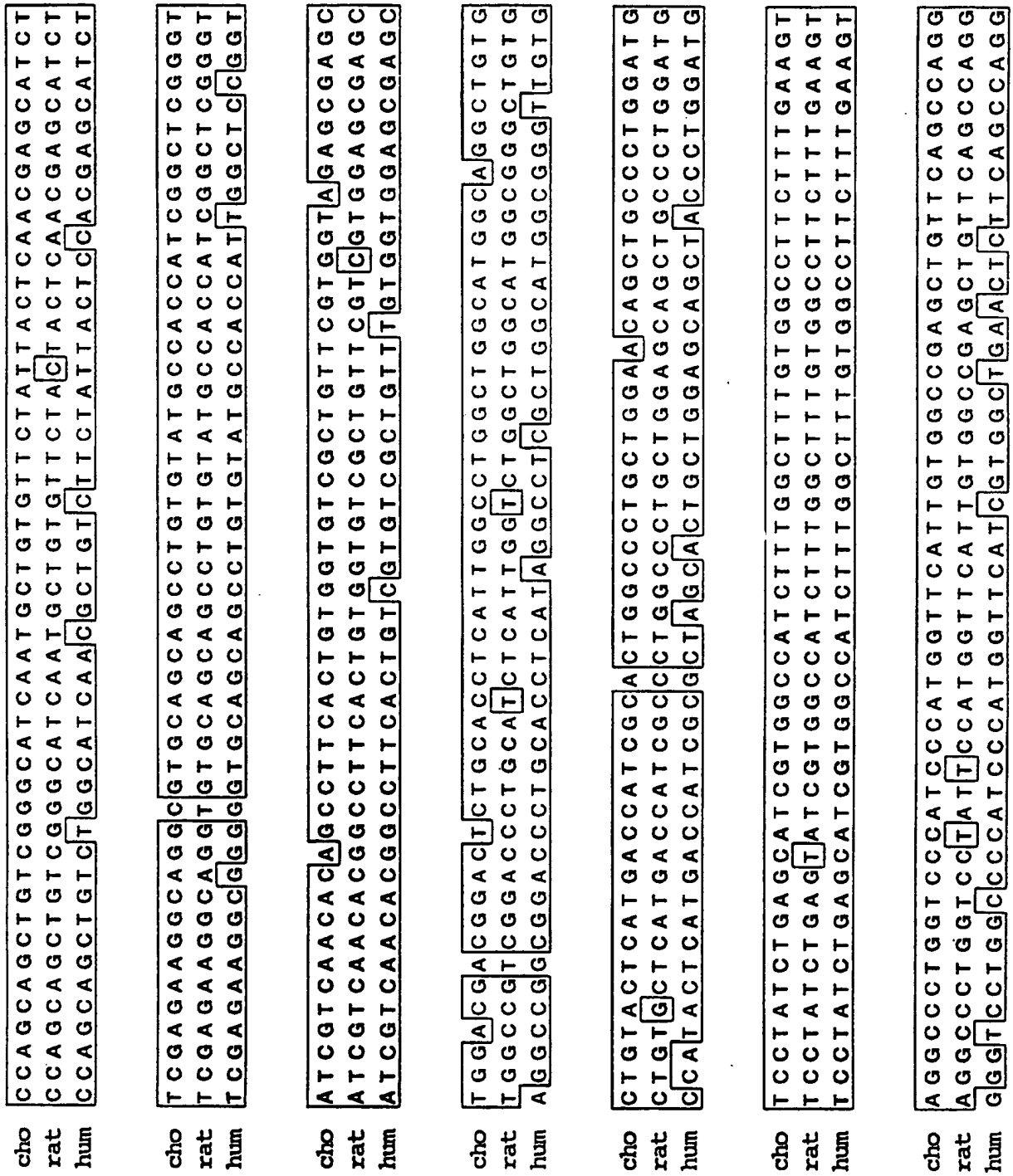
cho CCAGCAGCTGTCGGGCATCAATGCTGTGTTCTATTACTCAACGAGCATCT
rat CCAGCAGCTGTCGGGCATCAATGCTGTGTTCTACTACTCAACGAGCATCT
hum CCAGCAGCTGTCTGGCATCAACGCTGTCTTCTATTACTCCACGAGCATCT
cho TCGAGAAGGCAGGCGTGCAGCAGCCTGTGTATGCCACCACCATCGGCTCCGGGT
rat TCGAGAAGGCAGGTGTGCAGCAGCCTGTGTATGCCACCACCATCGGCTCCGGGT
hum TCGAGAAGGCGGGGGTGCAGCAGCCTGTGTATGCCACCACCATTGGCTCCGGGT
cho ATCGTCAACACAGCCTTCACTGTGGTGTCGCTGTTCGTGGTAGAGCGAGC
rat ATCGTCAACACGGCCTTCACTGTGGTGTCGCTGTTCGTCGTGGAGCGAGC
hum ATCGTCAACACGGCCTTCACTGTCGTGTCGCTGTTTGTGGTGGAAGCGAAGC
cho TGGACGACGGACTCTGCACCTCATTGGCCCTGGCTGGCATGGCAGGCTGTG
rat TGGCCGTCGGACCCTGCATCTCATTGGTCTGGCTGGCATGGCGGGCTGTG
hum AGGCCGGGCGGACCCTGCACCTCATAGGCCTCGCTGGCATGGCGGGTTGTG
cho CTGTACTCATGACCATCGCCACTGGCCCCTGCTGGAACAGCTGCCCCTGGATG
rat CTGTGCTCATGACCATCGCCCCTGGCCCCTGCTGGAGCAGCTGCCCCTGGATG
hum CCATACTCATGACCATCGCGCTAGCACTGCTGGAGCAGCTACCCCTGGATG
cho TCCTATCTCTGAGGCATCGTGGCCATCTCTTTGGCTTTGTGGCCTTCTTTGAAGT
rat TCCTATCTCTGAGTATCGTGGCCATCTCTTTGGCTTTGTGGCCTTCTTTGAAGT
hum TCCTATCTCTGAAGCATCGTGGCCATCTCTTTGGCTTTGTGGCCTTCTTTGAAGT
cho AGGCCCCTGGTCCCATCCCATGGTTCATTGTGGCCCGAAGCTGTTTCAGCCCAGG
rat AGGCCCCTGGTCCCTATCCATGGTTCATTGTGGCCCGAAGCTGTTTCAGCCCAGG
hum GGGTCCTGGCCCCCATCCCATGGTTCATCGTGGCTGAACTCTCTTCAGCCCAGG

Fig.9D.

Fig. 9E.

```
cho GGCCTCGTCCTGCTGCTATTGCTGTGGCTTCTCCAACTGGACCTCA
rat GGCCCCGACCTGCTGCTGTTGCTGGCTTCTCTACTGGACCTCA
hum GTCCCACGTCCAGCTGCCATTGCCGTTGCAGGCTTCTCCAACTGGACCTCA

cho AATTTCATCGTGGGCATGTGCTTCCAGTATGTGGAGCAACTGTGTGGCCC
rat AACTTCATCGTGGGCATGTGCTTCCAATATGTGGAGCAACTGTGTGGCCC
hum AATTTCATTGTGGGCATGTGCTTCCAGTATGTGGAGCAACTGTGTGGTCC

cho CTATGTCTTCATCATCTTCACGGTGCTCCTGGTGCTGTTCTTCATCTTCA
rat CTACGTCTTCATCATCTTCACGGTGCTGCTGGTACTCTTCTTCATCTTCA
hum CTACGTCTTCATCATCTTCACTGTGCTCCTGGTTTCTGTTCTTCATCTTCA

cho CCTACTTCAAAGTTCCTGAGACCAAAGGCCGGACCTTCGATGAGATCGCT
rat CCTACTTCAAAGTTCCTGAGACCAAAGGCCGGACCTTCGATGAGATCGCT
hum CCTACTTCAAAGTTCCTGAGACTAAAGGCCGGACCTTCGATGAGATCGCT

cho TCTGGCTTCCGGCAGGGGGGTGCAAGCCAAAGTGACAAGACACCTGAGGA
rat TCCGGCTTCCGGCAGGGGGGTGCCAGCCAGAGCACAAGACACCTGAGGA
hum TCCGGCTTCCGGCAGGGGGGAGCCAGCAAAGTGATAAGACACCCGAGGA

cho GCTGTTCCACCCTCTGGGGGCTGACTCCCAAGTGTGAGGAGCCCCACACC
rat GCTCTTCCACCCTCTGGGGGCTGACTCCCAAGTGTGAGGAGCCCACAGCC
hum GCTGTTCCATCCCCTGGGGGCTGATTCCCAAGTGTGAGTCGCCCCAGATC

cho CAGCCCCGGCCTACTCCCAGCAGCCCCCAAGAATCTCTGGAGCAAAGG
rat AGTCCCGCCTGCTCCCAGCAGCCCCGAGGATCTCTCTGGAGCACAGG
hum ACCAGCCCGGCCTGCCCAGCCCTAAGGATCTCTCAGGAGCACAGG
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Fig. 9F.

Fig. 9G.

cho
rat
hum
cho
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cho
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hum
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cho
rat
hum
cho
rat
hum

Fig. 9H.

Fig.10A.

```
cho MEPSSKKVTGRLMLAVGGAVLGSLQFGYNTGVINAPQKVIEEFYNQTWVH
rat MEPSSKKVTGRLMLAVGGAVLGSLQFGYNTGVINAPQKVIEEFYNQTWNH
hum MEPSSKKLTGRLMLAVGGAVLGSLQFGYNTGVINAPQKVIEEFYNQTWVH

cho RYGEPIVPTTLTTLWSLSVAIFSVGGMIGSFSVGLFVNRFGRRNSMLMMN
rat RYGESIPSTTLTTLWSLSVAIFSVGGMIGSFSVGLFVNRFGRRNSMLMMN
hum RYGESILPTTLTTLWSLSVAIFSVGGMIGSFSVGLFVNRFGRRNSMLMMN

cho LLAFVSAVLMGFSKLGKSFEMLILGRFIIGVYCGLTTGFVPMYVGEVSPT
rat LLAFVSAVLMGFSKLGKSFEMLILGRFIIGVYCGLTTGFVPMYVGEVSPT
hum LLAFVSAVLMGFSKLGKSFEMLILGRFIIGVYCGLTTGFVPMYVGEVSPT

cho ALRGALGTLHQLGIVVGILIAQVFGLDSIMGNADLWPLLLSIIFVPALLQ
rat ALRGALGTLHQLGIVVGILIAQVFGLDSIMGNADLWPLLLSVIFIPALLQ
hum AFRGALGTLHQLGIVVGILIAQVFGLDSIMGNKDLWPLLLSIIFIPALLQ

cho CILLPFCPESPRFLLINRNEENRAKSVLKKLRGTADVTRDLQEMKEEGRQ
rat CILLPFCPESPRFLLINRNEENRAKSVLKKLRGTADVTRDLQEMKEEGRQ
hum CIVLPFCPESPRFLLINRNEENRAKSVLKKLRGTADVTHDLQEMKEESRQ

cho MMREKKVTILELFRSAAYRQPILIAVVLQLSQQLSGINAVFYYSTSIFEK
rat MMREKKVTILELFRSPAYRQPILIAVVLQLSQQLSGINAVFYYSTSIFEK
hum MMREKKVTILELFRSPAYRQPILIAVVLQLSQQLSGINAVFYYSTSIFEK

cho AGVQQPVYATIGSGIVNTAFTVVSLFVVERAGRRTLHLIGLAGMAGCAVL
rat AGVQQPVYATIGSGIVNTAFTVVSLFVVERAGRRTLHLIGLAGMAGCAVL
hum AGVQQPVYATIGSGIVNTAFTVVSLFVVERAGRRTLHLIGLAGMAGCAIL
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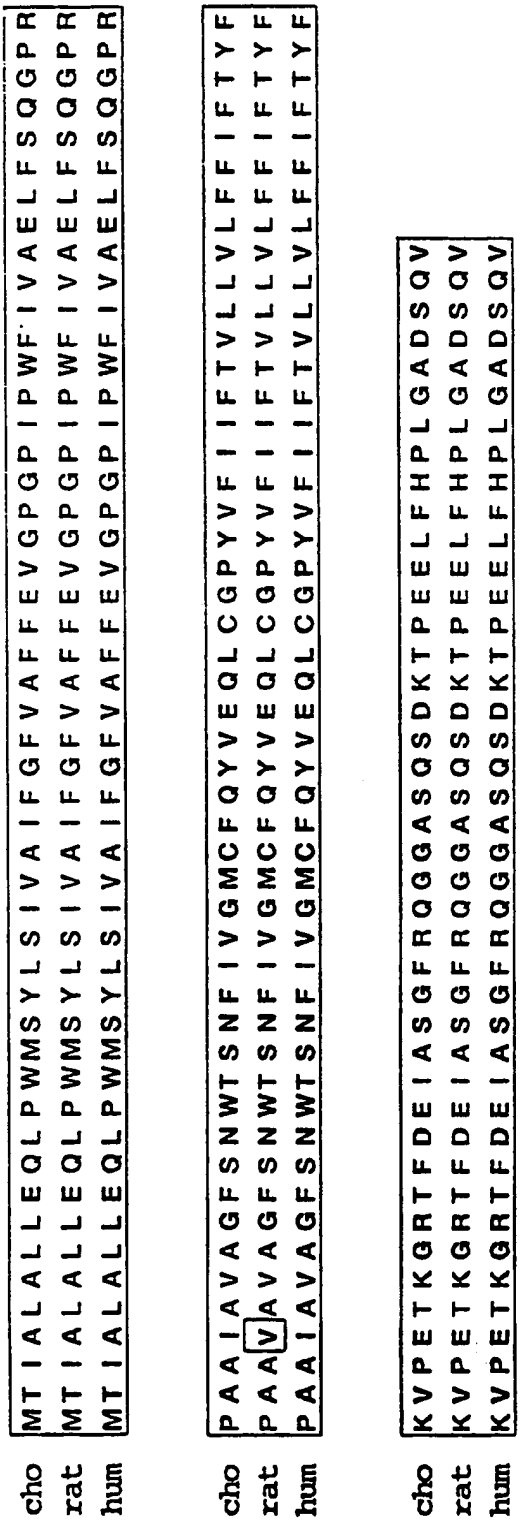
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rat MTIALALLEQLPWMSYLSIVAIFGFVAFFEVGPGPIPWFIVAELFSQGPR
hum MTIALALLEQLPWMSYLSIVAIFGFVAFFEVGPGPIPWFIVAELFSQGPR
cho PAAIAVAGFSNWTSNFIVGMCFQYVEQLCGPYVFIIFTVLLVLFFIFTYF
rat PAAVAVAGFSNWTSNFIVGMCFQYVEQLCGPYVFIIFTVLLVLFFIFTYF
hum PAAIAVAGFSNWTSNFIVGMCFQYVEQLCGPYVFIIFTVLLVLFFIFTYF
cho KVPETKGRTFDEIASGFRQGGASQSDKTPEELFHPLGADSQV
rat KVPETKGRTFDEIASGFRQGGASQSDKTPEELFHPLGADSQV
hum KVPETKGRTFDEIASGFRQGGASQSDKTPEELFHPLGADSQV

Fig.10B.

Fig.11A.

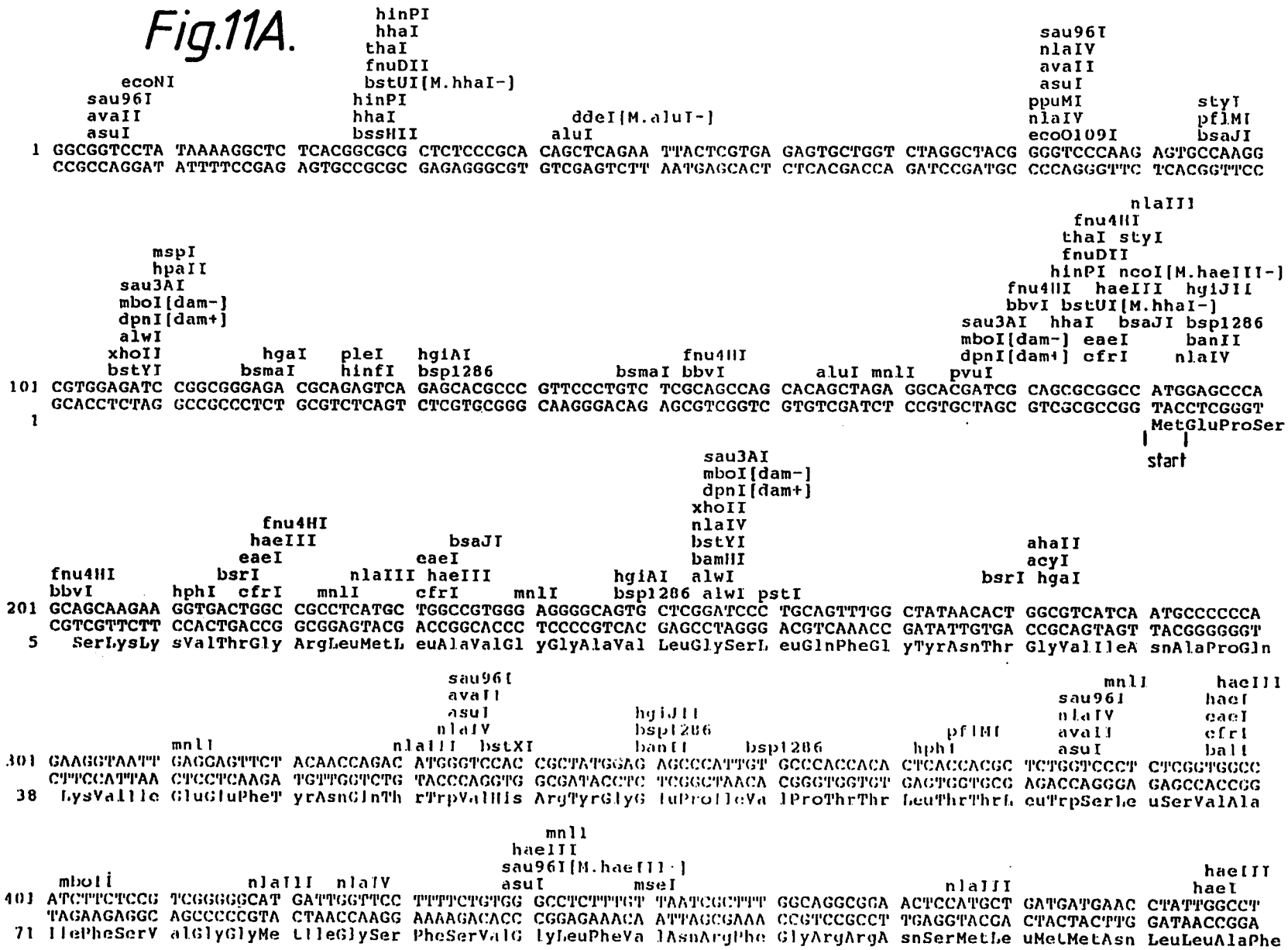
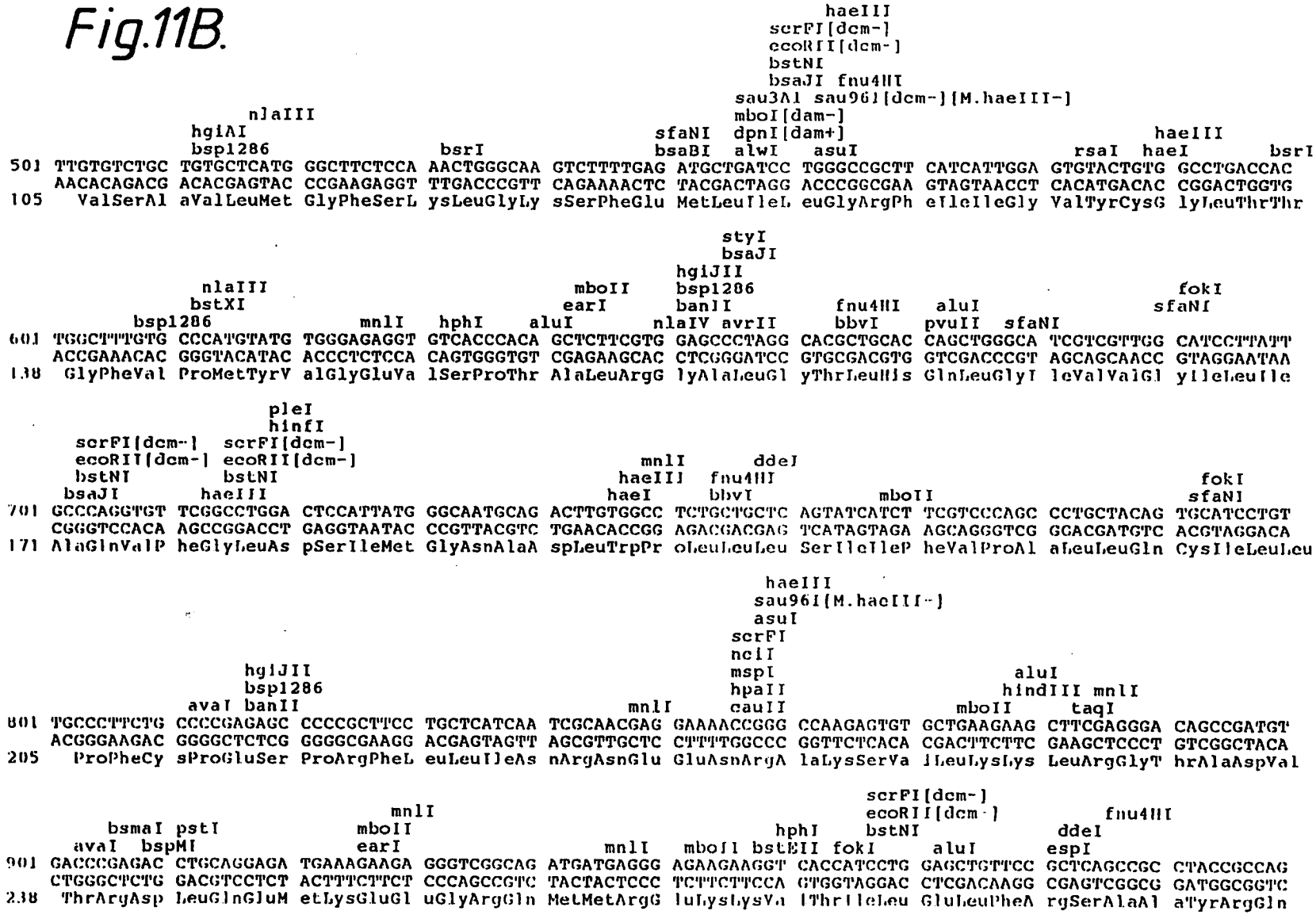


Fig.11B.



SUBSTITUTE SHEET

Fig.11C.

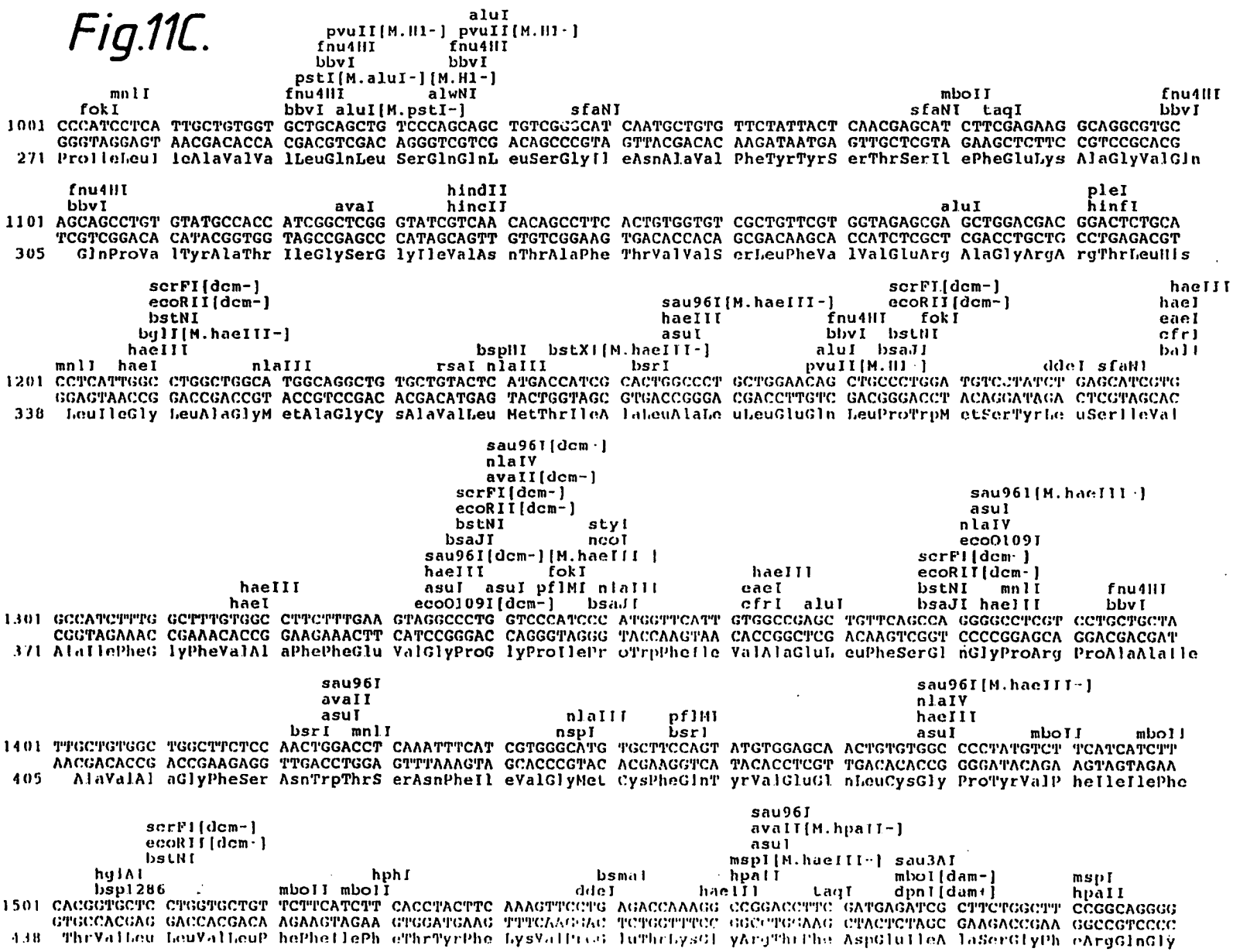


Fig.11D.

Fig.11E.

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                                                                                                                             scrFI[dcm-]
                                                                                                                             ecoRII[dcm-]
                                                                                                      haeIII      fnu4HI
         mseI               bsmaI                  bsmI                                               sau96I[M.haeIII-] bstNI
         aseI               ddeI   bsrI            bsrI           mnlI mnlI                           asuI      bsrI bbvI
2201 CCTTCCCAAC CACTGAATTA ATCTTTCCTT GCCTGAGACC AGTTGGAAGC ACTGGAATGC AGGGAGGAGA GGGGAAGGGC CAGACTGGGC TGCCAGGTTC
     GGAAGGGTTG GTGACTTAAT TAGAAAGGAA CGGACTCTGG TCAACCTTCG TGACCTTACG TCCCTCCTCT CCCCTTCCCG GTCTGACCCG ACGGTCCAAG

                                                  mnlI
                                                  bsaJI
                                                  bglI[M.haeIII-]
                  sau96I[M.haeIII-]               haeIII
           hgiAI  asuI                            sau96I[M.haeIII-]
           bsp1286 mnlI                           asuI                      mboII
   bsmaI   apaLI ddeI haeIII       hphI nlaIII eco0109I  mnlI ddeI
2301 TAGTCTCCTG TGCACTGAGG GCCAGACATC ACCATGAGAA GGGCCTCGGA GGCTGAGAAC TCACTGCTGA AGACACGGAC ACTCCTGCCC TGCTGTGTAT
     ATCAGAGGAC ACGTGACTCC CGGTCTGTAG TGGTACTCTT CCCGGAGCCT CCGACTCTTG AGTGACGACT TCTGTGCCTG TGAGGACGGG ACGACACATA

                                        mseI
      mboII                         sspI             ddeI
2401 AGATGGAAGA TATTTATATA TTTTTTGGTT GTCAATATTA AATACAGACA CTAAGTTATA GTATATCTGG ACAAACCCAC TTGTAAATAC ACCAACATAC
     TCTACCTTCT ATAAATATAT AAAAAACCAA CAGTTATAAT TTATGTCTGT GATTCAATAT CATATAGACC TGTTTGGGTG AACATTTATG TGGTTGTATG

                 ddeI
2501 TCCTGTAACT TTACCTAAGC AATATAAATG GCTGGTTTTT AGAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAA
     AGGACATTGA AATGGATTCG TTATATTTAC CGACCAAAAA TCTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTT
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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/04849

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12 ; C12N5/10 ; C12N15/85

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[9]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	CELL. vol. 55, 21 October 1988, CAMBRIDGE, MA US pages 281 - 290; THORENS, B. et al.: "Cloning and functional expression in bacteria of a novel glucose transport present in liver, intestine, kidney, and beta-pancreatic islet cel " see abstract	50
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 265, no. 10, 05 April 1990, BALTIMORE US pages 5793 - 5801; HARRISON, S.A. et al "Insulin action on activity and cell surface disposition of human HepG2 glucose transporters expressed in Chinese Hamster Ovary Cells" see the whole document	1-3, 5, 14, 17, 24-26, 33-35

[9] Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12 DECEMBER 1990	23 JAN 1991
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 264, no. 6, 25 February 1989, BALTIMORE US pages 3416 - 3420; ASANO, T. et al "Rabbit brain glucose transporter responds to insulin when expressed in insulin-sensitive Chinese Hamster Ovary Cells" see the whole document ---	1-3, 5, 14, 17, 24-26, 33-35